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14. ABSTRACT

Purpose: Single-arm studies have demonstrated preliminary signs of efficacy for intermittent pharmacologic dose testosterone (i.e. Bipolar Androgen Therapy; BAT) in treating advanced prostate cancer. In this project, we will conduct detailed molecular assessments on biospecimens (i.e. blood, metastatic tissue) from men receiving BAT to determine somatic and germline factors that predict for response/resistance. We will also evaluate additional PDT-based regimens (e.g. combinatorial treatments) in preclinical models.
Scope: This annual technical progress report details progress made during the first year of funding for this project (30 Sep 2016 – 1 Oct 2017).
Major Findings: During Year 1 we have focused on biospecimen acquisition from men enrolled a Phase II study testing BAT vs. enzalutamide. Results from correlative and preclinical studies are not available at this time.

15. SUBJECT TERMS

Castration-resistant prostate cancer, testosterone, bipolar androgen therapy, supraphysiologic testosterone, biomarker, mechanism of action

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9. INTRODUCTION:

The androgen receptor (AR) is frequently upregulated as PC adapts to a low androgen environment – likely driving resistance to AR-signaling inhibition. Interestingly, some adapted PC cell lines display blunted growth when exposed to high androgen levels, potentially due to the induction of dsDNA breaks and errors in DNA relicensing. We hypothesized that the adaptive autoregulation of AR may serve as a therapeutic liability, sensitizing PC cells to supraphysiologic testosterone (SPT) induced cell death. To explore this concept further, we designed a mode of SPT therapy termed Bipolar Androgen Therapy (BAT), whereby men with castration-resistant prostate cancer (CRPC) are treated intermittently with very high Pharmacologic Dose Testosterone (PDT). In a proof of concept study, we showed that BAT resulted in PSA and radiographic responses in ~50% of men. However, this study did not incorporate biospecimen acquisition that would have allowed us to determine the molecular events driving these clinical responses. Recently, a large randomized trial testing BAT *vs.* enzalutamide in men with CRPC was launched. As part of our participation in this study, we will obtain blood and metastatic biopsies from men receiving BAT in order to identify biomarkers that predict for response/resistance to BAT, and understand the mechanisms of action underlying these responses.

10. KEYWORDS:

Castration-resistant prostate cancer, testosterone, bipolar androgen therapy, supraphysiologic testosterone, biomarker, mechanism of action

11. ACCOMPLISHMENTS:

Major goals of the project:
Major goals of the project as indicated in the Statement of Work. Milestones/target dates for important subtasks, with completion dates or percentage of completion. Note: months are from the start of the funding period (9/30/2016).

Training-Specific Tasks:

Major Task: Training and educational development in prostate cancer research	**Months**	**Percent completed (date of completion)**
Subtask 1: Audit select courses: BIOST 517 and 518: *Applied Biostatistics;* GENOME 372: *Genomics and Proteomics;* and GENOME 552: *Technologies for Genome Analysis Present*	24-48	0%
Subtask 2: Attend UW training seminars: Institute of Translational Health Sciences Clinical and Translational Boot Camp; Series on Biomedical Research Integrity; Grant Training Series; Faculty Grants Management Workshop	1-18	25%
Subtask 3: Attend UW conferences/tumor boards: prostate cancer Precision Tumor Board; Genitourinary (GU) Tumor Board; Localized and Advanced GU Oncology Clinical Trials Conferences	1-48	25%
Subtask 4: Attend National Conferences/Committees: the Prostate Cancer Foundation Annual Retreat; the American Society of Clinical Oncology (ASCO) annual meeting; and ASCO GU annual meeting; Southwest Oncology Group meetings	1-48	25%

Major Task: Training and educational development in prostate cancer research	Months	Percent completed (date of completion)
Subtask 5: Present research at least once per year at Pacific Northwest Prostate Cancer SPORE research conferences	1-48	25%
Subtask 6: Provide direct care for patients with prostate cancer in my clinic. Supervise and educate medical residents and interns and medical oncology fellows	1-48	25%
Subtask 7: Prepare a grant submission to test a novel targeted therapy in the context of precision oncology trials	36-48	0%

Research-Specific Tasks:

Specific Aim 1: Identify somatic alterations in castration-resistant prostate cancers that associate with response and resistance to PDT and determine their causal roles in mediating treatment effects.

Major Task 1: Biospecimen Acquisition	Months	Percent completed (date of completion)
Subtask 1: Enroll ≥20 patients onto Phase II BAT *vs.* Enzalutamide Trial	1-24	70%
Subtask 2: Perform metastatic biopsies and collect blood samples from men receiving BAT	1-24	70%
Subtask 3: Isolate CTCs	1-24	70%
Subtask 4: Process plasma from ctDNA	1-24	70%
Major Task 2: Conduct Targeted Assays to Determine Somatic Features that Associate with Response to BAT		
Subtask 1: Conduct studies to assess for the presence of biallelic loss of DNA damage repair genes	24-40	0%
Subtask 2: Conduct studies to assess for alterations in AR at the genomic, transcript and protein levels	24-40	0%
Subtask 3: Conduct immunohistochemistry and immunofluorescence studies to assess for evidence of DNA damage and decreased cellular proliferation	24-40	0%
Subtask 4: Analyze results to determine if biallelic loss of DNA damage repair genes or alterations in AR associate with response to BAT	40-43	0%
Major Task 3: Conduct Molecular Profiling Studies to Determine Somatic Features that Associate with Response to BAT		
Subtask 1: Conduct transcriptome profiling (RNA-seq) studies on CTCs and metastatic tumors	24-40	0%
Subtask 2: Analyze transcriptome profiling results to assess for predictors of response to BAT	40-43	0%

Specific Aim 2: Evaluate the association of germ-line variations in genes contributing to AR activity and androgen metabolism with response and resistance to PDT.		
Major Task: Evaluate the impact of germ-line variations in androgen transport genes on response and resistance to BAT		
Subtask 1: Evaluate for the presence of germline *SLCO* polymorphisms using qRT-PCR	24-40	0%
Subtask 2: Perform liquid chromatography-mass spectrometry (LC/MS) assays on metastatic biopsy specimens to determine: intratumoral androgen (i.e. testosterone, DHT) and hormonal substrate (i.e. DHEA-S) levels	24-40	0%

Specific Aim 3: Conduct preclinical studies designed to augment the effectiveness of PDT including dosing schedules, testosterone concentrations, and drug combinations.		
Major Task: Conduct preclinical studies to evaluate different PDT schedules, testosterone concentrations and drug combinations		
Subtask 1: Establish LNCaP95 xenografts in castrated SCID-17B mice	12-18	0%
Subtask 2: Evaluate the effect of different PDT schedules, testosterone concentrations and drug combinations on LNCaP95 xenograft growth	18-30	0%
Subtask 2: Perform immunohistochemical studies to evaluate the effects of PDT on AR-FL, AR-SV and γ-H2AX.	18-30	0%

Year 1 Research Accomplishments:

The project entails conducted detailed molecular assessments on biospecimens obtained from men enrolled to a Phase II study testing bipolar androgen therapy (BAT) *vs.* enzalutamide. As outlined in the Statement of Work, Year 1 has focused on biospecimen acquisition. Accomplishments from this funding period are provided below.

- Year 1 Objectives: Begin biospecimen acquisition for downstream cell-free circulating tumor DNA (ctDNA), circulating tumor cell (CTC) and tumor tissue analyses.

- Major Activities: We have enrolled 14 (70%) out of a projected 20 subjects to the Phase II BAT *vs.* enzalutamide study. Seven of these patients have received BAT to date, and we anticipate the others will cross over to the testosterone arm in the near future. Blood samples have been obtained from all 7 patients treated with BAT, and 3 patients have undergone metastatic biopsies (one patient underwent baseline and Day 8 biopsy). We have also received additional plasma samples from patients treated on BAT clinical trials from our collaborators at Johns Hopkins. Blood samples have been processed for downstream ctDNA and CTC analyses.

- Results and Outcomes: Blood and metastatic tissue collection is ongoing. Molecular assessments are planned to be completed in Years 3 and 4 of the project.

- Goals not Met: None.

Year 1 Training and Professional Development:
This Training Award includes a multi-dimensional plan designed to endow me with the skills and practical knowledge that will allow me to recognize, develop and effectively exploit new approaches for treating prostate cancer. This training plan involves both didactic and 'hands-on' professional development experiences. Experiences from Year 1 are outlined below.

- Seminars and Conferences: As outlined in the Statement of Work, participation in a number of seminars is ongoing through the end of this Training Award. I continue to attend and present at the weekly Pacific Northwest Prostate Cancer SPORE conference series, as well as attend the weekly Oncology Center grand rounds. I regularly attend the Prostate Cancer Precision Tumor Board and GU Tumor Board, and actively participate in our Localized and Advanced GU Oncology Clinical Trials Conferences. In addition, I have attended the Faculty Grants Management Workshop.
- National Conferences and Committees: This past year I have attended the annual ASCO meeting, the ASCO GU meeting and the Prostate Cancer Foundation annual retreat. I have also attended the Spring 2017 Southwest Oncology Group (SWOG) meeting and presented a concept for a trial there.
- Clinical Development: I see and manage men with localized and advanced prostate cancer on an outpatient basis 1.5 days per week. I also attend on the inpatient unit ~4-weeks per year. I receive clinical management advice from Dr. Nelson, Dr. Yu and other senior faculty. In my clinic I actively enroll patients onto clinical trials, including the Phase II BAT *vs.* enzalutamide study described in this project.
- Mentoring/Training: I interact regularly with medical residents and oncology fellows, and have identified an Internal Medicine Resident interested in oncology research. Over the past year, I have mentored her by providing career advice, writing a letter of recommendation for her oncology fellowship application, and publishing a peer-reviewed article with her. I have also worked with a high-school student interested in oncology, and have written a case report with him.
- Lectures: I have delivered several lectures over the past year, including at the Weekly Fellows' Lecture Series, Seattle Cancer Care Alliance Comprehensive Hematology and Oncology Review Course and Department of Medicine Core Teaching Conference.
- Peer Review Activities: Over the past year, I have reviewed a number of articles for peer-reviewed journals. I also served on a grant review committee for the DOD PCRP.

Results Dissemination:

Nothing to report.

Funding Year 2 Plans:

Over the next reporting period, I plan to complete biospecimen acquisition as outlined in the Statement of Work. This will allow me to begin the molecular assessments described in Specific Aims 1 and 2. I will also begin preclinical studies (Specific Aim 3) to assess combinatorial PDT-based regimens.

4. IMPACT:

Nothing to report.

5. CHANGES/PROBLEMS:

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Note: Federal funding was only acknowledged for papers that were at least partly related to the project serving as the foundation for this award. All papers published within the reporting period are included for completeness.

1. **Schweizer, M.T.** & Yu, E.Y. AR-Signaling in Human Malignancies: Prostate Cancer and Beyond. Cancers 9(2017). Acknowledgement of Federal Funding: Yes
2. **Schweizer, M.T.**, Cheng, H.H., Tretiakova, M.S., Vakar-Lopez, F., Klemfuss, N., Konnick, E.Q., Mostaghel, E.A., Nelson, P.S., Yu, E.Y., Montgomery, B., True, L.D. & Pritchard, C.C. Mismatch repair deficiency may be common in ductal adenocarcinoma of the prostate. Oncotarget 7, 82504-82510 (2016). Acknowledgement of Federal Funding: No
3. **Schweizer, M.T.**, Antonarakis, E.S. & Denmeade, S.R. Bipolar Androgen Therapy: A Paradoxical Approach for the Treatment of Castration-resistant Prostate Cancer. European urology 72, 323-325 (2017). Acknowledgement of Federal Funding: Yes
4. **Schweizer, M.T.** & Antonarakis, E.S. Prognostic and therapeutic implications of DNA repair gene mutations in advanced prostate cancer. Clinical advances in hematology & oncology : H&O 15, 785-795 (2017). Acknowledgement of Federal Funding: No
5. Guedes, L.B., Antonarakis, E.S., **Schweizer, M.T.**, Mirkheshti, N., Almutairi, F., Park, J.C., Glavaris, S., Hicks, J., Eisenberger, M.A., De Marzo, A.M., Epstein, J.I., Isaacs, W.B., Eshleman, J.R., Pritchard, C.C. & Lotan, T.L. MSH2 Loss in Primary Prostate Cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 23, 6863-6874 (2017). Acknowledgement of Federal Funding: No
6. Chi, E.A. & **Schweizer, M.T.** Durable Response to Immune Checkpoint Blockade in a Platinum-Refractory Patient With Nonseminomatous Germ Cell Tumor. Clinical genitourinary cancer 15, e855-e857 (2017). Acknowledgement of Federal Funding: No

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Michael Schweizer*
Project Role:	*PI*
eRA Commons User Name:	*mschwei9*
Nearest person month worked:	*6*
Contribution to Project:	*Coordinates all aspects of the research in this project, including: planning, data gathering and analysis.*
Funding Support:	*DoD PCRP PRTA (W81XWH-16-1-0484)*

Name:	*Peter Nelson*
Project Role:	*Mentor*
eRA Commons User Name:	*pnelson*
Nearest person month worked:	*1*
Contribution to Project:	*Provides advice and resources to ensure this project is carried our as described.*
Funding Support:	*PNW Prostate Cancer Spore (P50 CA097186-12)*

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Updated Support Document for the PI (Dr. Schweizer) is attached as an appendix.

Other organizations involved as partners:

Organization Name: Johns Hopkins School of Medicine
Location of Organization: Baltimore, MD
Partner's contribution to the project

- *Provided biospecimens from men receiving bipolar androgen therapy (BAT).*

8. APPENDICES:

cancers

MDPI

Review

AR-Signaling in Human Malignancies: Prostate Cancer and Beyond

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Abstract: In the 1940s Charles Huggins reported remarkable palliative benefits following surgical castration in men with advanced prostate cancer, and since then the androgen receptor (AR) has remained the main therapeutic target in this disease. Over the past couple of decades, our understanding of AR-signaling biology has dramatically improved, and it has become apparent that the AR can modulate a number of other well-described oncogenic signaling pathways. Not surprisingly, mounting preclinical and epidemiologic data now supports a role for AR-signaling in promoting the growth and progression of several cancers other than prostate, and early phase clinical trials have documented preliminary signs of efficacy when AR-signaling inhibitors are used in several of these malignancies. In this article, we provide an overview of the evidence supporting the use of AR-directed therapies in prostate as well as other cancers, with an emphasis on the rationale for targeting AR-signaling across tumor types.

Keywords: prostate cancer; breast cancer; bladder cancer; renal cell carcinoma; pancreatic cancer; ovarian cancer; hepatocellular cancer; ovarian cancer; endometrial cancer; androgen receptor

1. Androgen Receptor Biology

Androgens, or male sex hormones, have a wide range of functions, including promoting the development of male secondary sexual characteristics, stimulating erythropoiesis, increasing metabolic rate, increasing bone density and stimulating libido [1]. In men, androgens are produced predominately by the testes, while the sole source of androgens in women are the adrenal glands. Consequently, women have considerably lower androgen levels compared to men. The normal physiologic function of androgens is a result of stimulating the androgen receptor (AR).

The AR is a member of the nuclear hormone receptor family of transcription factors, which also includes the estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and others [2,3]. Like the other nuclear hormone receptors, transcription of AR target genes is induced by the receptor binding androgenic ligands. Canonical AR-signaling involves a well-described series of events, including: (1) AR binding to androgens; (2) dissociating from heat-shock proteins; (3) translocating to the nucleus and the formation of AR homodimers; (4) binding to androgen response elements (AREs) within the promoter region of AR target genes; (5) recruitment of coactivators; and (6) transcription of target genes [4].

In addition to its normal physiologic role, prostatic adenocarcinomas remain dependent on AR-signaling even at later stages. Supporting the importance of AR to prostate cancer biology is the observation that AR target genes (e.g., *PSA*) are usually expressed even in men progressing on androgen deprivation therapy (ADT), with AR pathway alterations commonly observed in late stage

disease [5]. This has served as the basis for ADT through medical and surgical castration, as well as the development of next generation AR-directed therapies like abiraterone and enzalutamide.

As our understanding of AR biology has improved, it has become apparent that the AR-signaling pathway can interact with a number of additional oncogenic signaling pathways, including those involved in promoting growth and resistance across a variety of tumor types (e.g., AKT/mTOR/PI3K, EGFR, HER2/Neu, Wnt) [5–12]. Interestingly, in spite of differences in consensus DNA binding motifs, AR is able to bind estrogen response elements and activate a transcriptional program similar to the ER—indicating that AR may be important mediator of breast cancer cell survival as well as other ER-dependent tumors [13,14]. The pleiotropic effects of AR-signaling raise the specter that targeting this pathway may have beneficial effects in a number of different cancers. In this review, we will outline the current evidence for testing AR-directed therapies in prostate, breast and other "non-hormonally" driven cancer like bladder, renal cell and pancreatic cancer, to name a few.

2. AR Targeting in Prostate Cancer

In 1941, Charles Huggins published his seminal paper describing the remarkable palliative effects of surgical castration in men with advanced prostate cancer [15]. We now understand that the beneficial effects of castrating therapy are a direct result of inhibiting AR-signaling, and as such targeting the AR has remained the backbone of prostate cancer therapy since the 1940s. As it stands, ADT is most often achieved through the use of luteinizing hormone releasing hormone (LHRH) agonists/antagonists as opposed to surgical castration; however, both achieve the same effect of lowering testosterone levels to the castrate range (i.e., <20–50 ng/dL) [16]. While ADT is initially highly effective, it does not represent a cure, and the vast majority of men with advanced prostate cancer will progress on ADT, developing castration-resistant prostate cancer (CRPC) [17,18].

Work over the last decade has shown that the AR remains a viable therapeutic target even in the castration-resistant setting. This was born out of the observation that AR target genes (e.g., *PSA*) are often expressed at high levels in patients with CRPC, and that expression of AR will go up in response to ADT [19,20]. It has also come to light that alternative sources of androgens, including those generated intratumorally, may also drive tumor growth in this setting [21,22]. As such, a number of next-generation AR-directed therapies have been developed to further inhibit AR-signaling, with abiraterone and enzalutamide both approved on the basis of Phase III data demonstrating improved overall survival compared to controls [23–27]. Abiraterone is a CYP17 inhibitor that targets extragonadal androgen biosynthesis in the tumor microenvironment and adrenal glands. Enzalutamide is an AR antagonist that is more effective than the first generation non-steroidal antiandrogens (e.g., bicalutamide, nilutamide). Because both of these agents target the ligand-AR interaction—abiraterone through ligand depletion and enzalutamide through antagonizing the AR-ligand binding domain—it is not surprising that numerous groups have documented evidence of cross-resistance between these drugs [28–35].

More recently, a number of studies have described mechanisms whereby AR-signaling is able to reemerge in spite of treatment with next generation AR-signaling inhibitors. Examples of these mechanisms include: AR amplification/overexpression, intratumoral androgen production, activation via feedback pathways (e.g., AKT/mTOR/Pi3K, HER2/Neu), activating AR ligand binding domain mutation, emergence of constitutively active AR splice variants and activation through other nuclear hormone transcription factors (e.g., GR) [6,7,19,21,36–48]. Several in depth reviews of these mechanisms have been published, and a detailed overview of their role in promoting resistance to AR-directed therapies is beyond the scope of this paper [3,20,49]. Suffice it to say, many ongoing drug development efforts are focused on developing more effective AR-directed therapies (e.g., drugs *not* targeting the ligand-AR interaction like EPI-506) or drugs to target key feedback pathways in selected populations (e.g., Akt inhibitors in patients with PTEN loss) [50–52].

3. Breast Cancer

3.1. AR in Breast Cancer

Like prostate cancer, breast cancer is a hormonally regulated malignancy. Indeed, shortly following the discovery that surgical castration was effective in men with advanced prostate cancer, Charles Huggins began exploring oophorectomy and adrenalectomy (with hormone replacement) as treatments for advanced breast cancer [53]. It is worth noting, however, that the German surgeon Albert Schinzinger was first credited with proposing oophorectomy as a treatment for breast cancer in the late 19th century [54]. While most hormonal-based therapies for breast cancer involve inhibiting estrogen receptor (ER)-signaling in hormone receptor positive subtypes, it has recently come to light that AR-signaling is likely an important modulator of breast cancer cell survival and may also be a viable target [55,56].

Several lines of clinical data support the biologic importance of AR-signaling in breast cancer, although AR positivity has been found to have variable prognostic impact across studies. Vera-Badillo, et al. conducted a systemic review of 19 studies that assessed AR immunohistochemistry (IHC) in 7693 patients with early stage breast cancer and found AR staining present in 60.5% of patients; interestingly, AR positivity was associated with improved overall survival (OS) [57]. The authors also found that AR positivity was more common in ER positive compared to ER negative tumors (74.8% vs. 31.8%, $p < 0.001$). However, it should be noted that AR antibodies used across studies was not consistent, nor was the cutoff defining "positivity", making it difficult to draw firm conclusion regarding the overall prevalence of AR positivity across breast cancer subtypes.

Another study analyzing AR expression from tissue microarrays (TMAs) of 931 patients reported that 58.1% stained positive for AR, and that the association of AR with improved OS was only true for patients with ER positive tumors [58]. Apocrine tumors (ER negative, AR positive) with HER2 positivity associated with poorer survival, while AR did not appear to impact OS in triple negative breast cancer (TNBC) cases. A study by Choi and colleagues focused specifically on TNBCs ($n = 559$), found that AR was expressed in 17.7% of these cases, and that AR positivity was a negative prognostic feature. Two subsequent meta-analyses found that AR expression associated with better outcomes across tumor subtypes, however (i.e., ER positive, ER negative, and TNBC) [59,60].

3.2. Targeting AR in Breast Cancer

As mentioned, AR and ER are both nuclear hormone transcription factors and share a number of similar biologic features [55]. Upon binding their respective ligands, they undergo conformational changes, dissociate from heat shock proteins, dimerize and bind to DNA response elements where they promote transcription of target genes [3,61]. A number of studies have documented mechanisms whereby crosstalk between AR and ER exists, with most evidence supporting a model in which AR inhibits ER signaling through a variety of mechanisms—providing a biological basis for why AR positivity may associate with improved outcomes in ER positive breast cancers. AR is able to compete with ER for bindings at ER response elements (EREs), and transfection of MDA-MB-231 breast cancer cells with the AR DNA binding domain has been shown to inhibit ER activity [13]. Because the transcriptional machinery of both ER and AR involves a number of shared coactivator proteins, AR also likely inhibits ER activity through competing for binding of these cofactors [62,63]. Interestingly, there is also evidence that AR and ER can directly interact, with the AR N-terminal domain binding to the ERα ligand binding domain leading to decreased ERα transactivation [64].

The biologic action of AR in ER-negative breast cancers may differ significantly. AR is expressed in 12% to 36% of TNBCs, and in contrast to ER-positive breast cancers, data suggests that AR may be able to drive progression in some ER-negative cell lines [65–71]. Supporting the biologic importance of AR, and its viability as a therapeutic target, preclinical data has shown that AR antagonists (e.g., bicalutamide, enzalutamide) exert an anti-tumor effect in a number of ER-negative breast cancer models [65,67,72].

AR positive TNBCs are generally referred to as molecular apocrine tumors; however, more recent work has defined TNBCs on the basis of their molecular phenotype [73,74]. Work by Lehmann and colleagues have defined six subtypes of TNBC on the basis of their gene expression profiles: basal-like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor (LAR) [74]. Interestingly, in spite of being ER-negative, the LAR subtype shares a gene expression signature similar to the luminal, ER-positive breast cancers. Chromatin immunoprecipitation (ChIP)-sequencing studies demonstrate that AR-binding events are similar to those of ERα in ER-positive breast cancer cell lines, indicating that AR may be able to substitute for ER in this context [14].

It should be noted that in addition to LAR tumors, other ER-negative, AR-positive breast cancer subtypes are sensitive to the effects of androgens [65,67]. Ni and colleagues have shown that in HER2-positive, ER-negative cell lines, AR mediates activation of Wnt and HER2 signaling in a ligand-dependent manner [67]. Further speaking to the importance of AR across breast cancer subtypes, Barton and colleagues reported that the next-generation AR antagonist enzalutamide is effective in several non-LAR TNBC subtypes. Interestingly, it has been shown that constitutively active AR splice variants (AR-Vs)—a well-described resistance mechanism in prostate cancer—are present in a large subset of breast cancer tumors, and that treatment of MDA-MB-453 cells (ER/PR-negative, HER2-negative, AR-positive) with enzalutamide can lead to the induction of AR-Vs [75]. The fact that a well-known resistance mechanism to AR-directed therapy appears relevant to breast cancer provides further support for the importance of AR-signaling in breast cancer.

3.3. Clinical Trials Targeting AR-Signaling in Breast Cancer

Early clinical data reported by Gucalp and colleagues supported AR as a therapeutic target in AR-positive, ER-negative/PR-negative breast cancers [76]. They conducted a single-arm, Phase II study testing bicalutamide 150 mg daily in patients with >10% nuclear AR staining. The primary endpoint was clinical benefit rate (CBR) defined as complete response (CR), partial response (PR) or stable disease >6 months. Overall, 51 of 424 (12%) screened patients were AR-positive as defined by the study. Twenty-eight patients were treated per protocol, with only 26 being evaluable for the primary endpoint. The study reported a clinical benefit in five patients (all with stable disease), which exceeded the predefined threshold (CBR = 4/28 patients) needed to justify further study.

A single-arm Phase II study testing enzalutamide in AR-positive TNBCs was more recently reported [77]. The primary endpoint was the CBR in "evaluable" patients which were defined as those with $\geq$10% AR staining and a response assessment. After testing 404 patient samples, 55% were found to have AR staining in $\geq$10% of cells. 118 patients were treated with enzalutamide, and 75 were "evaluable". Of the evaluable patients, the CBR at 16 and 24 weeks was 35% and 29% respectively. The median progression free survival (PFS) in this group was 14 weeks. In patients with an AR gene signature (n = 56), clinical outcomes were numerically improved compared to the overall "evaluable" group and those lacking the gene signature (N = 62)—suggesting that further refinement of predictive biomarkers beyond AR IHC is necessary.

Table 1. Ongoing studies testing AR-directed therapies in breast cancer. Abi, abiraterone; Enza, enzalutamide; AR, androgen receptor; AE, adverse event; MTD, maximum tolerated dose; CR, complete response; PR, partial response; and SD, stable disease.

Indication	Therapeutic Agent(s)	Disease State	Study Phase	Sample Size	Primary Endpoint	NCT Number
Breast cancer	Enza, enza + anastrozole, enza + exemestane, enza + fulvestrant	Advanced	Phase I	101	Safety	NCT01597193
Breast cancer	Enza + exemestane	Advanced	Phase II	247	Progression free survival	NCT02007512
Triple-negative breast cancer	Enza + paclitaxel vs. placebo + paclitaxel	Advanced	Phase III	780	Progression free survival	NCT02929576
AR positive, triple-negative breast cancer	Enza + taselisib	Advanced	Phase I/II	73	MTD	NCT02457910
AR positive, triple-negative breast cancer	Enza + paclitaxel	Localized (neoadjuvant)	Phase II	37	Pathologic complete response and minimal residual disease	NCT02689427
HER2 positive and AR positive breast cancer	Enza + trastuzumab	Advanced	Phase II	80	Clinical benefit rate: combined CR, PR and SD	NCT02091960
AR positive, triple-negative breast cancer	Enza	Localized (adjuvant)	Phase II	200	Treatment discontinuation rate	NCT02750358
AR positive, triple-negative breast cancer	Enza	Advanced	Phase II	118	Clinical benefit rate: combined CR, PR and SD	NCT01889238
Breast cancer	VT-464	Advanced	Phase I/II	110	MTD	NCT02580448
Breast cancer	Abi	Advanced	Phase I/II	74	MTD, causality of AEs, and clinical benefit rate: combined CR, PR and SD	NCT00755885
ER positive HER2 negative breast cancer	Abi	Advanced	Phase II	299	Progression free survival	NCT01381874
HER2 negative breast cancer	Abi	Advanced	Phase II	31	Clinical benefit rate: combined CR, PR and SD	NCT01842321
ER positive HER2 negative breast cancer	Abi vs. anastrozole	Localized (neoadjuvant)	Phase II	–	Gene expression differences	NCT01814865
AR positive breast cancer	Orteronel	Advanced	Phase II	86	Response rate: complete and partial responses	NCT01990209
Breast cancer	Orteronel	Advanced	Phase I	8	Safety, recommended Phase II dose, and decrease in estradiol levels	NCT01808040

Abiraterone, an inhibitor of extragonadal androgen biosynthesis, has also been tested in breast cancer [78]. In a randomized Phase II trial, abiraterone was compared to the aromatase inhibitor exemestane or the combination. In contrast to the aforementioned studies, this study focused on ER-positive patients and did not require positive AR staining in order to enroll. The authors cited two reasons for not mandating AR-positivity: (1) upwards of 80% of ER-positive breast cancers are also positive for AR; and (2) inhibition of CYP17 will also decrease estrogen levels. The primary endpoint was PFS. A total of 297 patients were randomized between treatment arms, with 102 receiving exemestane, 106 receiving exemestane plus abiraterone and 89 receiving abiraterone. Of note, enrollment to the abiraterone monotherapy arm was discontinued early after a pre-specified analysis determined that futility conditions had been met. After a median follow up of 11.4 months, there was no difference in median PFS between when abiraterone was compared to exemestane (3.7 vs. 3.7 months, $p = 0.437$), or when abiraterone plus exemestane was compared to exemestane (4.5 vs. 3.7 months, $p = 0.794$). Of note, there was also no difference in PFS in the subset of patients with AR-positive disease.

Given that some studies have shown signs of activity for AR-signaling inhibitors, a number of additional trials are either planned or underway testing AR-directed therapies in breast cancer patients (Table 1). However, it seems likely that these agents will only be effective in a subset of patients, and as such, the development of predictive biomarkers will be critical. Whether the AR will prove to be a clinically important target in breast cancer remains to be seen, but evidence to date does support further testing of drugs designed to inhibit this oncogenic pathway.

4. Other Tumor Types

In addition to prostate and breast cancer, there are a number of other malignancies in which AR-signaling appears to play a role in driving tumor growth. As such, there are several ongoing clinical trials testing AR-directed therapies across an array of cancer types (Table 2). A brief overview of the rationale for targeting AR in these malignancies is provided below.

4.1. Bladder Cancer

In 2016, it is estimated that 58,950 American men will be diagnosed with bladder cancer compared to only 18,010 women [79]. Even after controlling for environmental risk factors (e.g., tobacco exposure) men still have a 3–4-fold increased risk of developing bladder cancer [80–82]. The observed epidemiologic differences in bladder cancer risk between the sexes points to the potential for sex steroid pathways to play a role in the pathogenesis of this disease [83]. Women have also been found to have a worse prognosis compared to men after adjusting for stage at presentation, further bolstering the case that underlying biologic differences between the sexes influencing outcomes [84].

Androgen receptor has been found to be variably expressed in urothelial carcinoma specimens, with AR staining present in 12% to 77% of patients [85–89]. In general, AR expression appears comparable in men and women [85,86]. There is no clear relationship between AR expression and clinical outcomes, and gene expression profiling studies do not demonstrate a clear relationship between AR expression levels and The Cancer Genome Atlas (TCGA) subtype [86,90,91].

Preclinical studies evaluating the effect of androgens and AR-signaling on urothelial carcinoma tumorigenesis have found that AR-signaling may promote tumor formation. In vitro siRNA studies have found that AR knockdown can lead to decreased tumor cell proliferation and increased apoptosis, possibly mediated through AR's effect on *cyclin D1*, *Bcl-x(L)* and *MMP-9* gene expression [92]. In a separate set of experiments, mice engineered to not express AR in urothelial cells were found to have a lower incidence of bladder cancer following exposure to the carcinogen BBN [*N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine] [93]. In vitro experiments found that this effect may be due to modulation of p53 and DNA damage repair. Studies have also implicated AR in modulating various other oncogenic signaling pathways (e.g., EGFR, ERBB2, β-catenin), offering more evidence for the importance of AR-signaling as it pertains to bladder cancer biology [94,95].

Table 2. Ongoing studies testing AR-directed therapies in cancers other than breast or prostate cancer. Enza, enzalutamide; AR, androgen receptor; and MTD, maximum tolerated dose.

Indication	Therapeutic Agent(s)	Disease State	Study Phase	Sample Size	Primary Endpoint	NCT Number
Endometrial cancer	Enza + carboplatin + paclitaxel	Advanced	Phase II	69	Safety/objective tumor response	NCT02684227
Hepatocellular carcinoma	Enza vs. placebo	Advanced	Phase II	144	Overall survival	NCT02528643
Hepatocellular carcinoma	Enza vs. Enza + sorafenib	Advanced	Phase I/II	73	Safety	NCT02642913
Non-muscle invasive bladder cancer	Enza	Localized (chemoprevention)	Phase II	50	Recurrence rate	NCT02605863
Bladder cancer	Enza + cisplatin + gemcitabine	Advanced	Phase I	24	MTD	NCT02300610
AR positive ovarian cancer	Enza	Advanced	Phase II	58	Response rate: complete and partial responses	NCT01974765
Pancreatic cancer	Enza + gemcitabine + nab-paclitaxel	Advanced	Phase I	38	MTD	NCT02138383
Renal cell carcinoma	Enza	Localized (neoadjuvant)	Pilot/Phase 0	20	Cell proliferation and tumor apoptosis	NCT02885649
Mantle cell lymphoma	Enza	Advanced	Pilot/Phase 0	20	Response rate: complete and partial responses	NCT02489123
AR positive salivary cancer	Enza	Advanced	Phase II	45	Response rate: complete and partial responses	NCT02749903

Kawahara and colleagues recently published a paper describing a series of in vitro and in vivo experiments in AR-positive and AR-null bladder cancer models [96]. They found that DHT increased AR-positive bladder cancer cell line viability and migration in culture, while AR antagonists (i.e., hydroxyflutamide, bicalutamide and enzalutamide) inhibited viability and migration. Similarly, apoptosis was decreased following exposure to DHT, and anti-androgens had the opposite effect. Importantly, enzalutamide was found to inhibit AR-positive bladder cancer xenograft growth in vivo. On the basis of these findings, two clinical trials have opened to test enzalutamide in patients with bladder cancer. One is testing enzalutamide monotherapy as a chemoprevention strategy in patients with non-muscle invasive bladder cancer [clinicaltrials.gov: NCT02605863], and the other is testing it in patients with advanced bladder cancer in combination with gemcitabine plus cisplatin [clinicaltrials.gov: NCT02300610].

4.2. Renal Cell Carcinoma

Androgen receptor is expressed in the distal and proximal tubules of normal kidneys and is expressed in approximately 15% to 42% of renal cell carcinomas (RCC) [97–99]. IHC studies correlating AR expression with clinical outcomes have not been consistent, with some reporting an association with decreased survival, while others have found that AR expression was correlated with a favorable pathologic stage and an overall favorable prognosis [97,100,101].

In a study evaluating AR transcript levels using real-time PCR, it was found that AR mRNA expression levels correlated with pathologic T stage and cancer specific survival. Multivariate regression analysis found AR transcript levels were independently associated with cancer specific survival. Of note, AR mRNA levels did not differ between sexes.

A more recent analysis of the TCGA data revealed that high AR protein and transcript levels was associated with improved overall survival in patients with clear cell RCC (the most common pathologic subtype), but not other histologic subtypes of RCC (i.e., papillary or chromophobe) [102]. Interestingly, in clear cell RCC cases they found that AR mRNA expression did not differ between men and women, but that AR protein expression was significantly higher in men. The authors concluded that AR might function as a tumor suppressor in this context.

In vitro experiments have reported that exposure to DHT causes proliferation in AR-positive RCC cells, while enzalutamide can reduce cell viability [103]. Other groups have found that AR may mediate tumor growth through activating HIF-2α/VEGF-signaling [104]. Preclinical studies have shown that enzalutamide can inhibit RCC cell migration and invasion by modulating HIF-2α/VEGF expression at the mRNA and protein levels. A neoadjuvant Pilot study testing enzalutamide in RCC patients is currently underway, with the primary goal to determine the effects of enzalutamide on RCC apoptosis and cellular proliferation [clinicaltrials.gov: NCT02885649].

4.3. Pancreatic Cancer

Although the incidence of AR expression is not well defined in pancreatic cancer, AR does appear to be expressed [105]. A number of in vitro/in vivo studies have tested the effects of antiandrogens and/or androgen deprivation in pancreatic cancer models, and have, for the most part, shown that inhibiting AR-signaling exerts anti-tumor effect [106–113]. Preclinical work has demonstrated that this effect may be mediated through IL-6, with a model whereby IL-6 activates AR-signaling via STAT3 and MAPK. Importantly, IL-6 has been shown to enhance pancreatic cell migration, an effect that is blocked through AR knockdown with an AR siRNA [114].

Greenway reported the results of a randomized trial comparing flutamide (a non-steroidal antiandrogen) vs. placebo (n = 49) in patients with both localized and metastatic pancreatic cancer [115]. It should be noted that histologic confirmation of pancreatic cancer was not required, and 32 included subjects were diagnosed on the basis of clinical presentation/imaging studies. This trial reported a median survival of 226 vs. 120 days in the flutamide and placebo groups, respectively (p = 0.079,

Wilcoxon; $p = 0.01$, log-rank). Several other studies in patients with pancreatic cancer have not shown hormonal therapies to be beneficial, however [116–121].

Preliminary results from an ongoing Phase I study testing enzalutamide in combination with gemcitabine and nab-paclitaxel in patients with metastatic pancreatic cancer have recently been reported [122]. They have treated 19 patients, and report that 37% had tumor tissue positive for AR. Among 15 evaluable patients, two had a partial response and 13 had stable disease. Pharmacokinetic (PK) analyses did not find any evidence that enzalutamide altered the PK of either chemotherapeutic agent. Whether enzalutamide will prove to be an effective treatment for pancreatic cancer remains to be seen.

4.4. Hepatocellular Carcinoma

Androgen receptor appears to be expressed in subset of hepatocellular carcinomas (HCC), although, like pancreatic cancer, the incidence has not been well defined [123–126]. The majority of studies show that AR-positivity is associated with worse outcomes, including decreased progression free and overall survival as well as increased tumor size [126–129]. Studies have also linked AR-signaling with increased risk of developing hepatitis B and C related HCC [130–133]. AR has been found to promote HCC growth, migration and invasion in several preclinical studies, possibly through increasing oxidative stress and DNA damage, as well as suppressing p53 [134–136]. In vitro and in vivo studies targeting AR with either AR-siRNA or ASC-J9 (an AR protein degrader) resulted in decreased tumor growth [134]. A randomized Phase II study testing enzalutamide vs. placebo in HCC is currently underway [clinicaltrials.gov: NCT02528643].

4.5. Ovarian Cancer

In 1998, Risch hypothesized that epithelial ovarian cancers may develop as a result of androgens stimulating epithelial cell proliferation, and as it stands, a number of lines of evidence support the role for AR-signaling in the pathogenesis of the disease [137,138]. AR is highly expressed in ovarian cancers, with approximately 44% to 82% of tumors staining positive for AR [139–141]. Polycystic ovarian syndrome (PCOS), and its resultant hyperandrogenic state, are associated with hyperplastic and metaplastic changes in the surface epithelium of the ovaries, and women with ovarian cancer are more likely to have a history of PCOS compared to control cases [142,143]. The use of exogenous androgens (i.e., danazol, testosterone) has been associated with a >3-fold increased risk of developing ovarian cancer [144]. Preclinical models also support the hypothesis that androgens play a role in the development of epithelial ovarian cancers, with a number of oncogenic signaling pathways implicated in this process (e.g., TGF-β, IL-6/IL-8, EGFR) [138,145–147]. However, as it stand, the prognostic impact of AR expression in epithelial ovarian cancers is not clear [138].

A handful of clinical trials testing AR-signaling inhibitors in women with ovarian cancer have been completed, with no clear signs of activity. A single-arm Phase II study testing flutamide in ovarian cancer patients progressing on platinum chemotherapy has previously been reported [148]. Out of 68 women enrolled, only two objective responses (one complete and one partial response) were observed. In a second single-arm Phase II study, flutamide was given to 24 ovarian cancer patients who failed chemotherapy and only one partial response was observed [149]. Finally, in a single-arm Phase II study, Levine and colleagues treated 35 women with ovarian cancer who were in second or greater complete remission with bicalutamide and goserelin (LHRH agonist) [150]. This trial failed to meet the pre-specified metric to justify further studies testing this regimen, which was arbitrarily set at median PFS >13.5 months. More recent preclinical work has shown that enzalutamide is able to significantly inhibit the growth of ovarian cancer xenografts [151]. On this basis, a Phase II study has been launched to test enzalutamide in women with AR-positive, advanced ovarian cancer [clinicaltrials.gov: NCT01974765].

4.6. Endometrial Cancer

Similar to prostate and breast cancer, endometrial cancers are hormonally dependent, and hormonal agents targeting ER-/PR-signaling are options for select patients [152]. Given the similarities to breast and prostate cancer, Tangen and colleagues sought to explore the potential for targeting AR-signaling in advanced endometrial cancer [153]. They found that the majority of hyperplastic endometrial specimens evaluated (93%) had evidence of AR expression. This number decreased in primary tumors, and high-grade tumors (i.e., grade 3) were found to express less AR than low-grade tumors (i.e., grade 1) (53% vs. 74%). Metastatic specimens from 142 patients revealed AR expression in 48% of samples. On multivariate analyses, AR status did not provide additional prognostic value, however. Short-term cell culture experiments demonstrated that cell proliferation was inhibited by enzalutamide, and stimulated by the synthetic androgen R1881, providing justification for a Phase II study testing enzalutamide in combination with carboplatin and paclitaxel [clinicaltrials.gov: NCT02684227].

4.7. Mantle Cell Lymphoma

Mantle cell lymphoma shows a male predominance, and interestingly, male sex appears to associate with higher mortality based on a retrospective SEER analysis [154]. While it is not clear what underlies the poor outcomes in men with mantle cell lymphoma, AR is expressed across an array of hematopoietic cells, and may account for gender differences in the function of platelets and the immune system [155–157]. Furthermore, in contrast to other lymphomas, *AR* appears to be hypomethylated in mantle cell lymphoma—indicating that epigenetic silencing of *AR* gene expression may not be present in mantle cell lymphoma [158,159]. To our knowledge, large studies examining AR protein expression in mantle cell lymphoma samples have not been conducted. On the basis of these observations a pilot study was recently launched to assess the clinical effects of enzalutamide in patients with mantle cell lymphoma [clinicaltrials.gov: NCT02489123].

4.8. Salivary Gland Cancer

AR is expressed in the majority of lacrimal gland ductal carcinomas, and as a result AR staining is often used as part of the workup to confirm the diagnosis [160–166]. To date, there have been a handful of case reports/series documenting favorable outcomes in patients with salivary gland cancers treated with AR-directed therapies. A small case series (n = 10) reported a clinical benefit when ADT—most often single agent bicalutamide—was given to patients with salivary ductal carcinoma, with 50% of patients experiencing clinical benefit (i.e., stable disease, n = 3; partial response, n = 2) [167]. A case report has also reported favorable outcomes when ADT was combined with radiation therapy in a patient with AR-positive salivary gland cancer [168]. A single arm Phase II study testing enzalutamide in AR-positive salivary gland cancers is ongoing [clinicaltrials.gov: NCT02749903].

5. Conclusions

AR signaling is involved in a number of normal physiologic processes, and there is varying levels of evidence for its role in promoting cancer growth and progression across an array of malignancies. To date, prostate cancer remains the only malignancy with Level 1 evidence supporting the use of AR-directed therapies as an integral part of its treatment paradigm. However, mounting preclinical, epidemiologic and early phase clinical trial data support the further exploration of these drugs in diseases as varied as breast and salivary gland cancers, and it is likely that in the ensuing decade next generation AR-directed drugs will extend their reach beyond prostate cancer.

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Mismatch repair deficiency may be common in ductal adenocarcinoma of the prostate

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ABSTRACT

Precision oncology entails making treatment decisions based on a tumor's molecular characteristics. For prostate cancer, identifying clinically relevant molecular subgroups is challenging, as molecular profiling is not routine outside of academic centers. Since histologic variants of other cancers correlates with specific genomic alterations, we sought to determine if ductal adenocarcinoma of the prostate (dPC) – a rare and aggressive histopathologic variant – was associated with any recurrent actionable mutations. Tumors from 10 consecutive patients with known dPC were sequenced on a targeted next-generation DNA sequencing panel. The median age at diagnosis was 59 years (range, 40–73). Four (40%) patients had metastases upon presentation. Archival tissue from formalin-fixed paraffin-embedded prostate tissue samples from nine patients and a biopsy of a metastasis from one patient with castration-resistant prostate cancer were available for analysis. Nine of 10 samples had sufficient material for tumor sequencing. Four (40%) patients' tumors had a mismatch repair (MMR) gene alteration (*N* = 2, *MSH2*; *N* = 1, *MSH6*; and *N* = 1, *MLH1*), of which 3 (75%) had evidence of hypermutation. Sections of the primary carcinomas of three additional patients with known MMR gene alterations/hypermutation were histologically evaluated; two of these tumors had dPC. MMR mutations associated with hypermutation were common in our cohort of dPC patients. Since hypermutation may predict for response to immune checkpoint blockade, the presence of dPC may be a rapid means to enrich populations for further screening. Given our small sample size, these findings require replication.

INTRODUCTION

Precision oncology entails therapeutic decision-making on the basis of an individual patient's molecular tumor profile. To that end, it is imperative to develop strategies to rapidly identify clinically relevant patient subgroups. While next-generation sequencing technologies have greatly advanced molecular classification, they are not routinely used for prostate cancer and may be costly. Because histological variants can correlate with genomic alterations in other malignancies (e.g. colorectal carcinoma, acute myelogenous leukemia), we hypothesized that distinct prostate cancer histologies may also associate with underlying molecular aberrations – allowing for the rapid identification of patients for further screening [1–5]. In this study, we sought to determine if ductal prostate cancer (dPC) was associated with clinically actionable molecular features.

Ductal prostatic adenocarcinomas (dPC) are an aggressive histopathologic variant of prostate

cancer, characterized by large glands lined by tall, pseudostratified, columnar neoplastic epithelial cells [6]. Approximately 3% of all prostate cancers have at least a component of ductal histology, with only 0.2% having pure ductal histology [7]. Clinically, dPCs tend to have a more aggressive course – behaving similarly to Gleason 4 + 4 = 8 carcinomas [8]. Tumors with >10% ductal component are associated with a higher stage, are more likely to present with metastatic disease, and may be less responsive to androgen deprivation [7].

While the more aggressive clinical course associated with dPC has been well documented, little is known about the molecular features underlying this histologic subtype. Studies using fluorescence in situ hybridization have reported the prevalence of *TMPRSS2:ERG* fusions in ductal cases to range from approximately 10–50%, which is not substantially different than typical acinar carcinomas [9, 10]. Otherwise, gene expression profiling studies reveal extensive similarities between ductal and acinar adenocarcinomas. In one study comparing the transcriptional profile of eight ductal tumors to 11 acinar adenocarcinomas, differences in gene expression profiles encompassed only 25 genes [11].

Given that little is known regarding the underlying genomic abnormalities associated with the ductal histologic phenotype, we sequenced consecutive cases of dPC using the UW-OncoPlex platform – a targeted next-generation sequencing panel that includes genes with actionable or potentially actionable mutations [12].

RESULTS

Patient characteristics

From January 2015 to April 2016, ten consecutive patients with dPC were identified and their tumors were sequenced (Figure 1). The median age at diagnosis was 59 years (range, 40 to 73). Four (40%) patients had metastatic disease at the time of presentation. Additional details regarding the patients included in this study and their tumor samples are provided in Table 1.

Sequencing results

To characterize the molecular features of dPC, we sequenced 10 prostate cancers with prominent dPC components: nine samples from FFPE archival tissue (radical prostatectomy or prostate needle biopsy specimens), and one frozen tissue biopsy from a metastasis. Nine of 10 samples had sufficient material for UW-OncoPlex testing. The tumors from four (40%) patients had an alteration predicted to be pathogenic in one of the mismatch repair (MMR) genes (2 in *MSH2*, 1 in *MSH6* and 1 in *MLH1*), of which 3 (75%) had evidence of hypermutation associated with microsatellite instability (MSI). The 3 patients with hypermutated tumors had evidence of bi-allelic MMR mutation. Other genomic alterations common to prostate cancer were also detected, including alterations in genes involved in homologous recombination repair (i.e. *BRCA2*, *CHEK2*) ($N = 2$), androgen receptor (*AR*) ($N = 1$), *TMPRSS2:ERG* rearrangements ($N = 3$) and alteration in the PI3K/Akt/mTOR signaling pathway ($N = 5$) (Table 2).

Histopathology of hypermutated prostate cancer

To determine the histopathologic features of hypermutated prostate cancer, we reviewed the pathology of known hypermutated cases from the University of Washington rapid autopsy program. We previously reported 5 prostate cancer patients who participated in this program and were found to have hypermutated tumors with complex MMR gene alterations [13]. Since that publication, we have identified 3 additional hypermutated prostate cancer cases using similar methods. Of the now 8 hypermutated prostate cancer cases in the autopsy series, 2 had untreated primary prostate cancer tissue available for pathology review. Both of these cases had a ductal adenocarcinoma component. The first subject (Autopsy Patient: 05-165) was previously reported to have an *MSH2-C2orf61* 343 kb inversion, *MSH2-KCNK12* 74 kb inversion, and *MSH2-KCNK12* 40 kb inversion [13]. The second subject (Autopsy Patient: 01-002), who was not included in our previous publication, had a germline *MSH2* exon 1–8 deletion with loss of heterozygosity in tumor tissue.

The tumor of a third patient with known hypermutated prostate cancer (determined through previously described methods) being followed in our clinic was histologically reviewed [14]. There was no ductal adenocarcinoma component in his tumor. It is notable, however, that this patient had a PSA decline following treatment with the immune checkpoint inhibitor pembrolizumab (i.e. anti-PD1) despite previously progressing on abiraterone, enzalutamide, docetaxel, carboplatin and cabazitaxel (Figure 2).

DISCUSSION

This series of consecutive patients with dPC represents the largest next-generation sequencing study focused on this rare prostate cancer subset to date. Consistent with other published reports, patients in our series had aggressive clinical features, including young age at diagnosis and a high proportion of metastatic disease at presentation [6–8, 15]. Surprisingly, we found that alterations in MMR genes and associated hypermutation were far more prevalent in dPC compared to prostate cancers not selected by histologic subtype [13, 14]. Providing further support for an association between ductal histology and MMR deficiency, we found that

two of three patients with MMR-deficient hypermutated metastatic prostate cancer whose primary tumors were available for review had dPC.

Hypermutated prostate cancers have only recently been described, with initial reported incidence ranging from approximately 3% to 12% in men with metastatic castration-resistant prostate cancer (mCRPC) [13, 14]. Although further validation to establish prevalence through larger systematic studies is needed, our findings are intriguing because they suggest a potential histologic association between the hypermutated genotype and a ductal histopathologic phenotype. More broadly, this finding supports an argument for sequencing rare histologic subtypes, as histology may provide insights into a tumor's underlying molecular features. Indeed, it is notable that a similar genotype-phenotype correlation in hypermutated MSI colorectal cancer has also been described – lending credence to the possibility that hypermutated cancers may have distinct histology compared to matched microsatellite stable cases [1, 3].

Determining which patients have hypermutated prostate tumors may have important implications for future precision oncology trials, as mutational burden has been shown to correlate with response to immune checkpoint blockade in several tumor types (e.g. anti-CTLA4, anti-PD1, anti-PDL1) [16–18]. Although objective responses to immune checkpoint inhibition have initially been generally disappointing in patients with prostate cancer, most have a relatively low mutational load [19, 20]. A recent Phase II study testing pembrolizumab (anti-PD1 therapy) in patients with metastatic colorectal carcinoma with and without MMR deficiency reported that 40% of hypermutated colorectal cancer patients had an immune-related objective response (irOR) compared to 0% of patients without MSI-high tumors. Moreover, a 50% response rate to pembrolizumab in hypermutated non-colorectal gastrointestinal malignancies has been observed – supporting the hypothesis that mutational load may be a predictive biomarker for response to immune checkpoint blockade in prostate cancer [17]. The observation that one of the hypermutated patients followed in our clinic had a dramatic response to anti-PD1 therapy in spite of being heavily pretreated further bolsters the hypothesis that hypermutation may be predictive of response to PD1/PDL1 pathway inhibition.

Consistent with our prior observations, we found that somatic loss-of-function mutations in *MSH2* and *MSH6* were the primary cause of microsatellite instability in patients with prostate cancer [13].This is in contrast to colorectal cancer where hypermutation has been found to be associated with epigenetic silencing of *MLH1*, which occurs in nearly 2/3 of the cases [21]. Interestingly, the tumor of Subject #2 showed evidence of *MSH2* inversion without clear evidence of hypermutation or MSI. Whether the *MSH2* loss-of-function alteration represents an early event and hypermutation is a later consequence in the disease course or follows selective pressures of treatment will need to be further examined. Given that the mechanisms underlying prostate cancer hypermutation appear distinct from colorectal cancer, patterns of MSI may also be divergent and a tailored approach to MSI testing of prostate cancer may be needed. However, our findings suggest that ductal histology may be a cue to investigate further for evidence of MMR deficiency and hypermutation.

The finding that prostate cancers with ductal histologic features may be enriched for somatic hypermutation is intriguing; however, our small sample size limits our ability to draw definitive conclusions regarding this genotype-histologic phenotype relationship. If this finding is confirmed, however, the presence of ductal

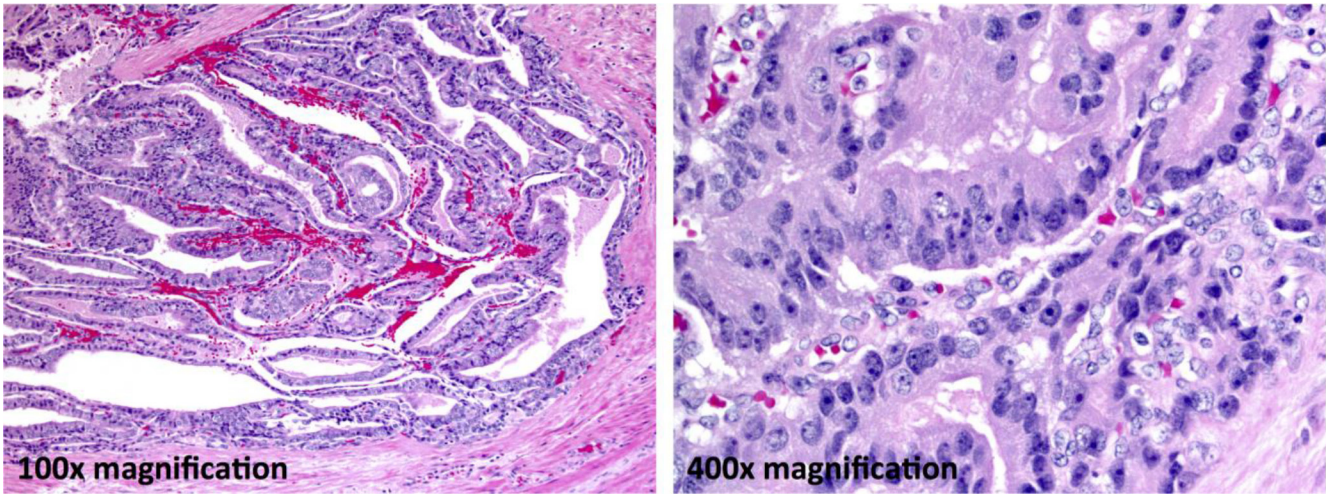

Figure 1: Ductal adenocarcinoma component. In this case, approximately 65% of the carcinoma is ductal. Large tumor cell aggregates have a tubulopapillary architecture (100× final magnification). Forming a pseudostratified columnar epithelium the tumor cells have markedly atypical nuclei with clumped chromatin and prominent nucleoli (400× final magnification).

Table 1: Demographics

Subject number	Age at diagnosis	Gleason	Disease state at presentation	Disease state at Time of Tissue Acquisition	Source of tissue for UW-OncoPlex	Clinical state at last follow up	Time from diagnosis to last follow up (months)
1	72	9	Localized	Localized	Prostatectomy	NED	34.8
2	69	9	Metastatic	mHSPC	Needle Biopsy	mHSPC	8.2
3	52	8	Localized	Localized	Prostatectomy	NED	16.6
4	66	9	Localized	Localized	Prostatectomy	Death	10.3
5	73	7	Localized	Localized	Prostatectomy	Biochemical recurrence	28.1
6	51	8	Localized	Localized	Prostatectomy	NED	28.7
7	40	9	Metastatic	mCRPC	Prostatectomy	mHSPC	1.0
8	61	9	Metastatic	mHSPC	Needle Biopsy	mHSPC	29.6
9	58	9	Localized	mHSPC	Needle Biopsy	NED	15.3
10	54	7	Metastatic	Localized	Soft Tissue Met	mCRPC	16.5

mHSPC, metastatic hormone-sensitive prostate cancer; NED, no evidence of disease; mCRPC, metastatic castration-resistant prostate cancer.

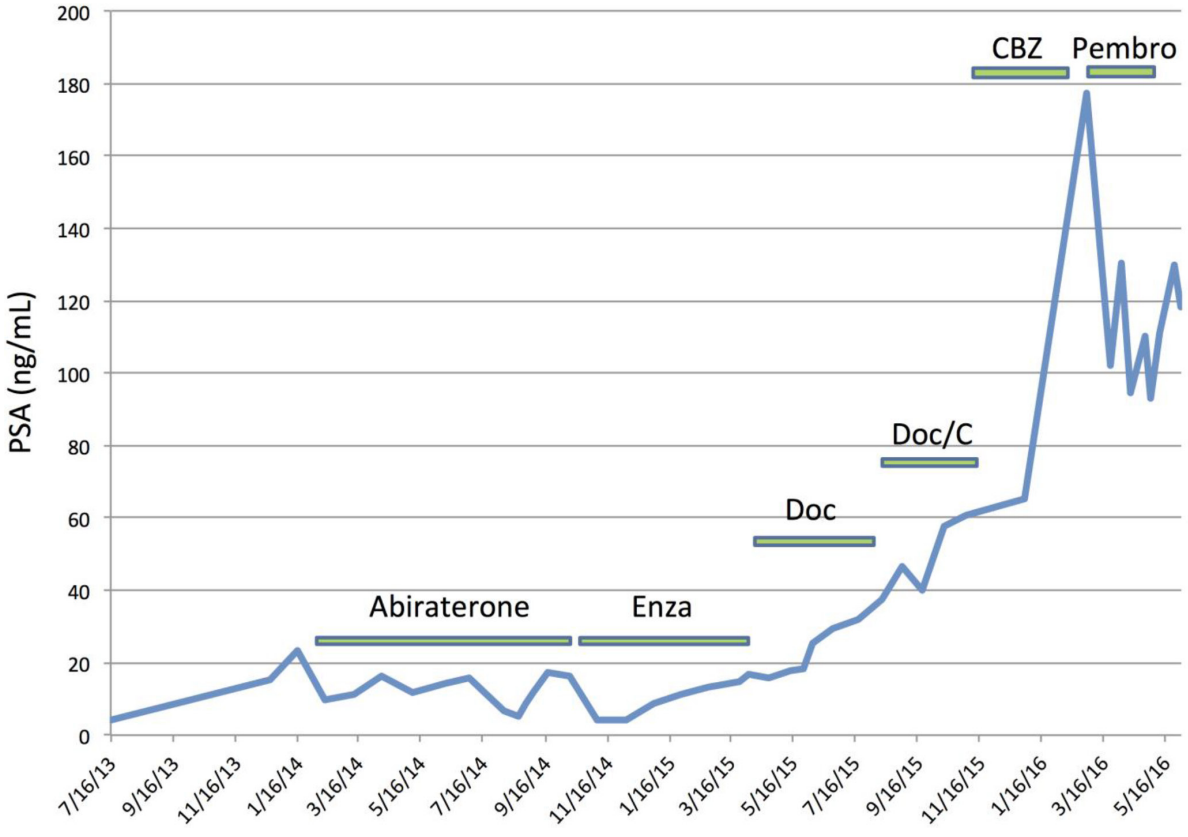

Figure 2: PSA response to checkpoint blockade immunotherapy in a patient with hypermutated prostate cancer. Prior to initiating pembrolizumab, this patient had bone, adrenal and lymph node metastases, and a baseline PSA of 177.35 ng/mL. A total of 3 cycles of pembrolizumab were administered before stopping due to an immune related adverse event (anasarca) requiring corticosteroids. He expired in June 2016. Note: this patient did not have ductal histopathologic features. Enza, enzalutamide; Doc, docetaxel; C, carboplatin; CBZ, cabazitaxel; Pembro, pembrolizumab.

Table 2: Summary of somatic alterations identified in ductal prostate cancer cases

Subject number	Ductal component of sample used for NGS	Tumor content estimated from NGS	MMR gene alteration	HR gene alteration	Hypermutated	Total Coding Mutations (per 1.2Mb sequenced)	Selected Other Mutations and Variants
1	71%	30%	No	*CHEK2* c.1100delC+LOH	No	4	*PIK3CA* p.H1047Y, *PIK3R1* p.R577del, *CDH1* p.P373L, *EPHA5* p.R896H
2	45%	40%	*MSH2* inversion	No	No	4	*TP53* p.L252_I254del, *FOXA1* p.S304R
3	65%	60%	No	No	No	4	*TMPRSS2:ERG* rearrangement, *PTEN* p.F90Lfs*9 (only in 4% of reads), IKZF1 p.E35K, ABL2 c.347-1G>T, PML p.V452M, and *TRRAP* p.E1229Q
4	30%	60%	*MSH6* c.1900_1901del+LOH	No	Yes	29	*PTEN c.*968dup
5	97%	50%	*MSH2-GRHL2* rearrangement +LOH	No	Yes	34	(Many frameshift mutations attributable to MSI)
6	99%	50%	No	No	No	5	*IDH1*p.R132C, *CTNNB1* (beta catenin) p.S33A, and *FOXA1* p.M253_F254del
7	25%	0%	–	–	–	–	Insufficient tissue for sequencing
8	31%	70%	No	No	No	5	*PTEN* copy loss, *TMPRSS2:ERG* rearrangement, *TP53* p.E258G
9	35%	10%	No	*BRCA2* c.5946delT+likely LOH	No	3	*SPOP* p.D130E, *FLT1* (VEGFR) rearrangement
10	-	60%	*MLH1* exon 19+ 3'UTR homozygous deletion	No	Yes	32	*AR* p.W742L, *PIK3CA* p.H1047R, *TMPRSS2:ERG* rearrangement, *FOXA1* rearrangement

All mismatch repair (MMR) gene and homologous recombination (HR) gene alterations were known or predicted to be pathogenic. Note: metastatic tissue from subject 10 was sequenced. LOH, loss of heterozygosity; NGS, next generation sequencing.

adenocarcinoma histology could be a means to prioritize patients for additional studies to assess mutational burden, which may have clinical implications, as hypermutation appears to predict for response to immune checkpoint blockade in several cancer types, including early signals in prostate cancer [22]. Future efforts to define the landscape of genomic alterations in patients with this prostate cancer variant will likely require multi-institutional studies. Such studies may facilitate the promise and rapid completion of precision oncology approaches for targeting this molecular subset of prostate cancer.

MATERIALS AND METHODS

Patients

All patients carried a diagnosis of prostate cancer and were followed by a medical oncologist at the University of Washington Medical Center or Seattle Cancer Care Alliance (both in Seattle, Washington). Consecutive patients with a component of ductal adenocarcinoma were identified by the treating medical oncologist and offered tumor sequencing. After obtaining written informed consent, tumor samples were tested on the UW-OncoPlex platform [12].The original diagnoses of dPC, made by genitourinary (GU) pathologists (M.S.T., F.V.L.), were independently verified by a third GU pathologist (L.T.).

Ethics statement

This study was performed in accordance with the declaration of Helsinki guidelines and with ethics approval from the Institutional Review Board at the Fred Hutchinson Cancer Research Center/University of Washington Comprehensive Cancer Consortium.

Macrodissection of tumor tissue

Hematoxylin and eosin stained sections of the tumors were reviewed by an anatomic and molecular pathologist. Ten-micron unstained recut sections were cut from the FFPE block, which were determined to contain the maximum amount of ductal adenocarcinoma. The dPC component, which ranged from 20% to 99% of the cells by visual estimate of each tumor, was macrodissected prior to deparaffinization and DNA extraction.

Next-generation sequencing (NGS) testing

DNA was extracted from FFPE samples as previously described [12]. Fresh tumor samples were snap frozen and unselected tissue was submitted for DNA extraction. UW-OncoPlex was performed according to previously published methods [12]. Microsatellite instability (MSI) testing was performed directly on NGS data using the mSINGS method [23]. Total mutation

burden was estimated from targeted NGS data as previously described, with hypermutation defined as > 12 mutations/megabase [24].

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Platinum Opinion

Bipolar Androgen Therapy: A Paradoxical Approach for the Treatment of Castration-resistant Prostate Cancer

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Since Huggins and Hodges first described the palliative benefits of surgical or medical castration in 1941, the treatment of advanced prostate cancer has focused almost exclusively on inhibiting androgen receptor (AR) signaling (ARS) [1]. Often overlooked, however, is the fact that Huggins also postulated that treatment with excess androgens, a strategy he called "hormone interference", could also produce a therapeutic benefit [2]. The seemingly paradoxical ability of supraphysiologic androgen levels to inhibit prostate cancer growth has been demonstrated in multiple in vitro and in vivo studies. In addition, a number of case series recounting the benefits of testosterone (T) supplementation in prostate cancer patients have peppered the literature for more than half a century [3–6]. More recently, preclinical studies have shed light on the mechanisms underlying the antitumor effects of androgens [7–11]. These findings have renewed our interest in exploring high-dose T as a therapeutic strategy for men with advanced prostate cancer, and have provided the impetus for the development of a series of prospective studies testing intermittent high-dose T in the clinic, a therapeutic strategy we have termed *bipolar androgen therapy* (BAT).

As prostate cancer cells transition from a hormone-sensitive to castration-resistant state, one of the most frequently observed events is adaptive upregulation of AR expression [12]. It has been shown that AR upregulation drives resistance to ARS inhibition. However, such upregulation may also create a therapeutic liability. We and others have observed that a number of AR-overexpressing cell lines display blunted cell growth and cell death when exposed to supraphysiologic androgen levels [8,13–19]. Thus, at supraphysiologic levels, T is able to exert a pharmaceutical effect in AR-overexpressing prostate cancer cells that results in inhibition of prostate cancer growth. Cells that adaptively downregulate AR expression or that have low basal levels of AR may also be killed when T levels are allowed to rapidly drop back to castrate levels over a cycle of BAT.

Studies exploring the mechanisms behind the paradoxical antitumor effects of supraphysiologic androgen levels have demonstrated that in high-AR cell lines, rapid transition from a castrate to a high-androgen environment induces transient double-strand DNA (dsDNA) breaks that can produce gene rearrangements such as *TMPRSS2-ERG* [7,11,13]. More recently, a clinical case report described an extreme response to BAT in a patient with germline mutations in the homologous recombination genes *BRCA2* and *ATM* [20].

Another potential mechanism underlying the antitumor effects of high-dose T is related to the role that AR plays as a DNA licensing factor in prostate cancer cells [9]. During the cell cycle, nuclear AR binds to origins of replication and participates in the formation of prereplicative complexes that allow DNA replication to proceed. Under normal conditions, AR is degraded from origins of replication and is absent during mitosis. Under high-androgen conditions, however, sufficient ligand-bound nuclear AR persists during mitosis, and probably interferes with DNA relicensing, leading to cell death in daughter cells.

Finally, differences in the AR transcriptome are present when high-AR cell lines are exposed to either high or low androgen concentrations [21]. Under high-androgen

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conditions, AR can repress a number of genes, including *AR* and those involved in androgen synthesis, DNA synthesis, and proliferation. Therefore, high-dose T may lead castration-resistant cells to transition from a more oncogenic transcriptome associated with castrate T levels to a high-androgen transcriptome that does not support cancer proliferation.

To exploit these mechanistic findings, we developed a mode of intermittent high-dose T therapy in the clinic termed BAT. We hypothesized that rapidly cycling between the polar extremes of near-castrate and supraphysiologic serum T (SPT) levels would prevent adaptive changes in AR expression, prolonging the length of time during which patients respond to this therapy. Furthermore, because recent studies have shown that the dsDNA breaks and apoptosis induced by high doses of androgens are transient, rapid cycling of T could result in repeated rounds of DNA damage, enhancing antitumor effects [22].

To date, BAT has yielded encouraging preliminary results in CRPC patients. In our first pilot study testing BAT combined with etoposide, we found that of the 14 patients completing at least the first 3 mo of therapy (response-evaluable cohort), 50% had PSA declines and 5/10 RESICT-evaluable patients had an objective soft-tissue response [13]. It is also notable that there was a high rate of response to subsequent ARS inhibitors (eg, abiraterone, antiandrogens), potentially indicating that BAT could effectively resensitize tumors to drugs inhibiting ARS. This observation provided justification for a study (NCT02090114; RESTORE) evaluating BAT in men after abiraterone or enzalutamide, with the co-primary endpoints of (1) response to BAT and (2) response to rechallenge with either abiraterone or enzalutamide. Preliminary results from the enzalutamide arm of the study have demonstrated a >50% PSA decline and an objective response in approximately one-third of patients. BAT was well tolerated, with low-grade musculoskeletal pain and breast tenderness being common side effects. On rechallenge with enzalutamide, ~50% of patients had a >50% PSA response. BAT is also able to suppress AR-V7 expression in most men with detectable AR-V7 in baseline CTC samples [23].

At present, BAT is being definitively tested in a large (n = 180) randomized trial (NCT02286921; TRANSFORMER) in asymptomatic CRPC patients who have failed on abiraterone. In this study, BAT is being compared to enzalutamide for the primary endpoint of progression-free survival. Patients are notably allowed to cross over following progression to the first therapy, with an important secondary endpoint being PSA progression-free survival on the second agent in order to further examine the question of the ability of BAT to resensitize tumors to ARS inhibitors.

As it stands, not all men respond favorably to treatment, and there is an urgent need to develop biomarkers able to discriminate between BAT responders and nonresponders. Candidate predictive biomarkers include high AR/AR-V7 expression and the presence of mutations in genes involved in DNA repair (eg, *BRCA1/2*, *ATM* and others). The case report of an extreme BAT responder with germline *BRCA2* and *ATM* mutations supports DNA damage as one potential mechanisms underlying response to high-dose T, while a second case report showing eradication of an *AR* copy gain (detected from ctDNA) and a clinical response to high-dose T supports AR levels as an important mediator of BAT's efficacy [20,24]. These hypotheses are actively being investigated in the RESTORE and TRANSFORMER trials.

Optimization of SPT-based therapies is still needed. While there is a strong rationale for an intermittent approach (ie, BAT), preclinical studies have demonstrated that continuous exposure to supraphysiologic androgen levels also has a robust antitumor effect. Clinical trials testing different dosing schedules are needed to determine if better modes for administration of SPT-based therapy exist. It also stands to reason that combinatorial SPT-based therapies may produce better outcomes. An emerging understanding of the mechanism of BAT inhibition have suggested that potential combinatorial strategies (eg, with PAPR inhibitors, platinum agents, proteasome inhibitors, immune checkpoint inhibitors) may be warranted, as are rational sequencing strategies (time-sequential therapies).

Conflicts of interest: The authors have nothing to disclose.

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Prognostic and Therapeutic Implications of DNA Repair Gene Mutations in Advanced Prostate Cancer

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Abstract: Recent work directed toward understanding the molecular features of advanced prostate cancers has revealed a relatively high incidence of both germline and somatic alterations in genes involved in DNA damage repair (DDR). Many of these alterations likely play a critical role in the pathogenesis of more aggressive prostate cancers—leading to genomic instability and an increased probability of the development of lethal disease. However, because the ability to repair DNA damage with a high degree of fidelity is critical to an individual cell's survival, tumor cells harboring alterations in DDR pathway genes are also more susceptible to drugs that induce DNA damage or impair alternative DNA repair pathways. In addition, because the genomic instability that results from these alterations can lead to an inherently higher number of mutations than occur in cells with intact DDR pathways, patients with genomic instability may be more likely to respond to immune checkpoint inhibitors, presumably owing to a correspondingly high neoantigen burden. In this review, we discuss the emerging molecular taxonomy that is providing a framework for precision oncology initiatives aimed at developing targeted approaches for treating prostate cancer.

Keywords
Checkpoint inhibitor, DNA damage repair, homologous recombination, mismatch repair, PARP inhibitor, prostate cancer

Introduction

Failure to repair DNA damage and replication errors accurately can lead to the accumulation of mutations and an increased risk for cancer. It is therefore not surprising that mutations in DNA repair genes have been associated with several cancer predisposition syndromes.[1-5] Studies across a variety of malignancies have also shown that when DNA damage repair (DDR) deficiency occurs—often as a result of homozygous loss-of-function mutations in *BRCA1/2*, *ATM*, and other genes involved in homologous recombination (HR)—intrinsic genomic instability is present, which can render cells vulnerable to agents that induce DNA damage or inhibit alternative DNA repair pathways. Poly(ADP-ribose) polymerase (PARP) has been shown to be a key mediator in this respect, and strategies to inhibit PARP activity have been shown to be effective in a number of cancers with impaired HR.[6-10] In addition, more

recent data have shown that targeting PARP activity may be an effective strategy to augment the antitumor effects of other DNA-damaging agents (eg, alkylating agents and platinum chemotherapeutic agents) in cancers with intact DDR pathways.[10,11] Tumors with homologous recombination deficiency (HRD) also appear to be exquisitely sensitive to DNA-damaging chemotherapeutic agents.[12,13]

In addition, because alterations in mismatch repair (MMR) pathway genes can lead to the accumulation of vastly more mutations than occur in tumors with an intact MMR pathway (ie, hypermutation), it has been hypothesized that such tumors will have a higher neoantigen burden, which renders them more susceptible to immune checkpoint inhibitors. A recent study testing this hypothesis has led to the first US Food and Drug Administration (FDA) tumor-agnostic approval for pembrolizumab (Keytruda, Merck) in patients with MMR gene mutations or microsatellite instability (MSI), a marker of genomic fragility.[14-16]

Alterations in the DDR pathway are present in upward of 20% of men with metastatic castration-resistant prostate cancer (mCRPC) and in up to 12% of men with metastatic prostate cancer harboring a germline alteration in one of these genes.[5,17] Given how prevalent these mutations are, it is not surprising that a number of precision oncology approaches are being developed to treat patients who have advanced prostate cancer with impaired DDR. This review outlines the clinically relevant DDR pathways as they pertain to prostate cancer and discusses efforts to develop drugs targeting these pathways.

DNA Damage Repair: Overview

A multitude of events occur daily that lead to DNA damage that requires subsequent repair. The ability to repair DNA damage with a high degree of fidelity is both critical to an individual cell's survival and necessary to prevent malignant transformation. As such, germline alterations in DDR genes can increase replicative DNA stress, the accumulation of mutations, and the risk for cancer.[18,19] Because of the critical role that DDR pathways play in maintaining cellular viability, a complex network of cellular pathways has evolved to deal with DNA damage by detecting and repairing it as it arises—herein referred to as DDR pathways.[20-23]

The DDR pathways are signal transduction pathways consisting of sensors, transducers, and effectors.[24,25] The ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) proteins are key kinases involved in sensing and regulating the response to DNA damage and are intimately involved in several DDR pathways.[26] If DNA damage is detected, cell cycle arrest occurs, providing an opportunity either for damaged DNA to be repaired via a number of DDR pathways or for apoptosis to occur if catastrophic genomic instability has occurred.[19,22,27] Some key proteins involved in regulating the cell cycle include the following: ATM (G1/S checkpoint), ATR (S-phase checkpoint), CHK1 (G2/M and S-phase checkpoints), CHK2 (G1/S checkpoint), DNA-PK (S-phase checkpoint), WEE1 (S-phase and G2/M checkpoints), and TP53 (G1/S checkpoint).[22]

Following the detection of DNA damage, overlapping downstream DDR pathways are activated to resolve double-strand DNA (dsDNA) damage or single-strand DNA (ssDNA) damage.[27] The key pathways involved in ssDNA repair are MMR, base excision repair (BER), and nucleotide excision repair (NER). The main pathways involved in dsDNA damage repair are HR and nonhomologous end joining (NHEJ).[28-38] A third pathway responsible for rescuing damaged dsDNA is called translesion DNA synthesis. Redundancies in these pathways ensure that even with loss-of-function mutations in one of these pathways, an individual cell may still be able to survive. Key proteins involved in these overlapping pathways are outlined in Table 1.[1,25,26]

Given the complexity of the DDR pathways, an exhaustive review of the topic is beyond the scope of this article. Instead, we focus on the pathways that appear most clinically relevant to the prognosis and treatment of prostate cancer.

Targeting Homologous Recombination Deficiency

Mutations in the genes involved in HR are frequently observed in men with metastatic prostate cancer.[5,17] Nearly 12% of unselected patients with metastatic prostate cancer have been found to have germline alterations in HR genes, and approximately 20% to 25% of patients with mCRPC harbor alterations in HR genes (somatic and/or germline), with *BRCA2*, *ATM,* and *BRCA1* the most commonly affected genes.[5,17] Studies examining the effect of germline *BRCA1/2* mutations on prostate cancer risk have reported that *BRCA2* confers an 8.6-fold increased risk for prostate cancer and that *BRCA1* confers a 3.4-fold increased risk.[39-43] *BRCA1/2* germline alterations have also been shown to be associated with a higher Gleason score, a higher T stage, nodal involvement, and metastases at diagnosis.[43] Rates of cause-specific overall survival and metastasis-free survival are also significantly lower for patients with localized prostate cancer and a germline alteration in *BRCA1* (hazard ratio, 2.6; P=.01) or *BRCA2* (hazard ratio, 2.7; P=.009).

The most genotoxic form of DNA damage is dsDNA damage because both strands of DNA are affected.[22,44] The 2 key pathways involved in resolving dsDNA damage

Table 1. Key DNA Damage Repair Pathway Sensors, Transducers, and Effectors

	dsDNA Repair Pathways			ssDNA Repair Pathways		
	HR	**NHEJ**	**alt-NHEJ**	**BER**	**MMR**	**NER**
Causes of damage	Radiation, topoisomerase I inhibitors, nucleoside analogues	Radiation, topoisomerase II inhibitors	Radiation, topoisomerase II inhibitors	Radiation, alkylating agents, oxidation, deamination	Replication/ recombination errors, alkylating agents	Ultraviolet light, polycyclic aromatic hydrocarbons, platinum chemotherapy
Sensors	MRN complex	Ku70-Ku80	PARP	APE1, 9-1-1 complex, PARP, RPA complex	MLH1, MSH2, MSH3, MSH6, PMS2	XPC, DDB2, CSA
Transducers	ATM, ATR, BRCA1, BRCA2, PALB2	ATM, DNAPK				RPA complex
Effectors	BLM, FANCJ, PARI, POLQ, RAD51, RECQL5	LIG4, PAXX, XLF, XRCC4	LIG1, LIG3, POLQ, XRCC1	LIG1, LIG3A, POLB, XRCC	EXO1, LIG1, POLD	ERCC1, POLE, POLD1, LIG 1, LIG 3, XPG

alt-NHEJ, alternative NHEJ; BER, base excision repair; dsDNA, double-strand DNA; HR, homologous recombination; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; ssDNA, single-strand DNA.

are NHEJ and HR. It is important to note that although HR results in error-free repair of dsDNA damage and uses the undamaged sister chromatid as a template, NHEJ is an error-prone repair mechanism that can lead to a large number of chromatid breaks and aberrations, which can result in loss of cell viability.[44,45] As mentioned earlier, HR is the major pathway for high-fidelity DNA repair following an insult that results in dsDNA damage. Cancers in which the tumor cells have biallelic loss-of-function mutations in genes involved in HR are sensitive to agents that induce DNA damage.

PARP Inhibitors in Prostate Cancer

PARPs (especially PARP1, PARP2, and PARP3) are key enzymes involved in BER and are required to repair ssDNA damage efficiently. Without PARP1 function, single-strand gaps in DNA persist, and degeneration to double-strand breaks can occur if a replication fork encounters these genomic defects.[45-48] Under normal conditions, such dsDNA damage can be repaired via the HR pathway; however, in the case of HRD, replication forks collapse and chromatid breaks persist, leading a cell down a pathway toward apoptotsis.[48-50] In addition, PARP1 is involved in repairing dsDNA breaks through the alternative NHEJ pathway and can therefore further impair the ability to repair dsDNA breaks in HR-deficient tumors.[51-53] Preclinical studies have supported this model, demonstrating that *BRCA1/2*-deficient cell lines are sensitive to pharmacologic PARP1 inhibition.[45,48]

Proof of concept for this approach is derived from TOPARP (A Phase II Trial of Olaparib in Patients With Advanced Castration Resistant Prostate Cancer).[6] This was a phase 2 study testing olaparib (Lynparza, AstraZeneca) at an oral dose of 400 mg twice daily in men with mCRPC. The primary endpoint was the response rate, which was defined as the presence of any of the following: an objective radiographic response per the Response Evaluation Criteria in Solid Tumors (RECIST) criteria v1.1, a reduction in the prostate-specific antigen (PSA) level of at least 50% from baseline (ie, a PSA_{50} response), or a confirmed reduction in the number of circulating tumor cells (CTCs) from at least 5/7.5 mL of blood to fewer than 5/7.5 mL of blood. Of the 50 patients with mCRPC who were enrolled, all had received prior docetaxel, and 49 had received prior abiraterone acetate (Zytiga, Janssen Biotech) or enzalutamide (Xtandi, Astellas/Medivation). There were 16 patients (33%) who met the primary endpoint, achieving a response according to the composite definition. Most notably, responses to olaparib were enriched in the subset of patients with loss-of-function alterations (homozygous deletions, deleterious mutations, or both) in HR genes (eg, *BRCA1/2*, *ATM*, Fanconi anemia genes, *CHK2*); the observed response rate was 88% in this biomarker-positive cohort. Interestingly, genomic reversions of germline and/or somatic DNA repair mutations that restore the open reading frame (ORF) were

described as driving secondary resistance in this trial.[54] Several subsequent studies have since been launched to evaluate PARP inhibitors further in men with recurrent or advanced prostate cancer (Table 2).

DNA-Damaging Agents

The induction of DNA damage is one of the most common mechanisms by which chemotherapeutic agents exert their cytotoxic effects. Given the importance of HR in repairing dsDNA damage, it is intuitive that cells with impaired HR activity will be sensitive to any number of DNA-damaging agents. Indeed, preclinical models have shown that *BRCA1* and *BRCA2* are important mediators of platinum-induced DNA damage, and loss of function of these genes can enhance platinum sensitivity.[45,55] Consistent with this finding is the observation that ovarian cancers with mutations in *BRCA1* or *BRCA2* are more susceptible to platinum chemotherapy.[56]

Several older trials that did not include next-generation sequencing of tumor samples tested platinum-based chemotherapy regimens in men with advanced prostate cancer.[57-61] Because most of these studies tested combination regimens, it is difficult to estimate the contribution of the platinum agent to the observed response rate. Many studies have reported PSA_{50} response rates of 15% to 30%—approximating the incidence of HRD in patients with CRPC.[17] A phase 2 study reported by Ross and colleagues is particularly informative. In that trial, the authors reported that of 34 men with CRPC that had progressed during or within 45 days of completion of docetaxel-based chemotherapy, 18% had a decline in PSA of at least 50% following treatment with docetaxel (60 mg/m^2) plus carboplatin (area under the curve [AUC], 4).[57] One can surmise that because this study enrolled only men with previously progression on docetaxel, the observed clinical effects were most likely the result of carboplatin activity.

Emerging data support HRD as a predictive biomarker for prostate cancer response to DNA-damaging agents. In a small case series, Cheng and colleagues reported on 3 heavily pretreated patients with mCRPC who had extreme responses to platinum-based chemotherapy; all of the men had deleterious alterations in HR genes.[12] Similarly, a recent retrospective analysis of patients with mCRPC who were receiving platinum-based chemotherapy revealed that PSA_{50} response rates were higher in men with known pathogenic germline *BRCA2* alterations. In this study, by Pomerantz and colleagues, 6 of 8 carriers (75%) of a pathogenic *BRCA2* variant had a PSA_{50} response following carboplatin plus docetaxel vs 23 of 133 men (17%) without a pathogenic *BRCA2* variant (P<.001).[62] On the basis of these data, a precision oncology trial testing docetaxel plus carboplatin in patients with mCRPC who have HRD was recently launched (NCT02598895).

Combination PARP Inhibitors and DNA-Damaging Agents

Because DDR inhibitors impair a cell's ability to resolve DNA damage, combining a PARP inhibitor with a conventional cytotoxic therapy could in theory potentiate the effects of the cytotoxic therapy. Consistent with this idea, PARP inhibitors have been shown across multiple preclinical tumor models to potentiate the antitumor effects of DNA-damaging cytotoxic agents (eg, alkylating agents, platinum chemotherapy) as well as of radiation.[63-67] Importantly, many of these studies have shown that the observed antitumor effects are not restricted to cell lines with a biallelic loss of HR pathway genes.

On the basis of preclinical work demonstrating synergy between PARP inhibitors and temozolomide, a number of trials testing PARP inhibitors in combination with temozolomide have been launched.[22] A pilot study testing low-dose veliparib with temozolomide in patients with mCRPC after docetaxel was previously reported by Hussain and colleagues.[68] Of the 26 patients eligible for this study, 25 were evaluable for PSA_{30} response (the primary endpoint). Overall, 2 of 25 patients (8%) had a confirmed PSA_{30} response, and there were no objective radiographic responses in the 16 patients with RECIST-evaluable disease. The authors questioned whether the low dose of veliparib (40 mg twice daily) tested in this trial could have affected the overall efficacy of the combination. In addition, temozolomide is not particularly active in prostate cancer and may not have yielded sufficient DNA damage in this tumor type. Somatic tumor sequencing was unfortunately not performed in this study, and the underlying HRD status of the enrolled subjects is not known.

The more recent I-SPY 2 trial (Neoadjuvant and Personalized Adaptive Novel Agents to Treat Breast Cancer) tested veliparib in combination with carboplatin as a neoadjuvant therapy in patients with breast cancer.[8] This study was a multicenter, randomized, phase 2 "platform" trial testing the addition of multiple experimental regimens to a control "backbone" regimen. Patients with high-risk primary breast cancer planning to undergo surgery were eligible. The control arm received 12 weekly cycles of paclitaxel followed by 4 cycles, every 2 to 3 weeks, of doxorubicin/cyclophosphamide. One of the experimental arms received a combination of 50 mg of veliparib by mouth twice daily and carboplatin (AUC, 6) concurrently with the weekly paclitaxel. The primary endpoint was the pathologic complete response (pCR) rate as assessed at the time of surgery. Among the patients with triple-negative breast cancer (ie, negative for human epidermal growth factor 2 [HER2], estrogen receptor [ER],

Table 2. Selected Ongoing Clinical Trials Testing PARP Inhibitors in Men With Prostate Cancer

Agents Being Tested	Trial Phase	Disease State	Key Eligibility Criteria	Sample Size	Primary Endpoint	Identifier
Olaparib +/- degarelix (Firmagon, Ferring Pharmaceuticals)	Phase 1	Localized	Intermediate- to high-risk disease Planning to undergo prostatectomy	20	Determination of PARP inhibition	NCT02324998
Olaparib +/- cediranib	Phase 2	mCRPC	Two or more prior lines of therapy for mCRPC	84	Radiographic PFS	NCT02893917
Rucaparib (Rubraca, Clovis Oncology)	Phase 2	mCRPC	HRD After taxane and 1-2 next-generation AR signaling inhibitors	160	Objective response rate PSA response rate	NCT02952534
Rucaparib vs abiraterone, enzalutamide, or docetaxel	Phase 3	mCRPC	HRD After next-generation AR signaling inhibitor	400	Radiographic PFS	NCT02975934
Niraparib (Zejula, Tesaro)	Phase 2	mCRPC	Progression on ≥1 taxane-based chemotherapy regimen and ≥1 AR signaling inhibitor	160	Objective response rate	NCT02854436
Niraparib + enzalutamide	Phase 1	mCRPC	—	—	MTD	NCT02500901
Olaparib	Phase 2	Biochemical recurrence	After prostatectomy Nonmetastatic disease	50	PSA response rate	NCT03047135
Olaparib + abiraterone	Phase 2	mCRPC	After docetaxel	159	Safety Radiographic PFS	NCT01972217
Abiraterone vs olaparib vs olaparib + abiraterone	Phase 2	mCRPC	HRD Before docetaxel	70	PFS	NCT03012321
Olaparib vs enzalutamide or abiraterone	Phase 3	mCRPC	HRD After abiraterone and/or enzalutamide	340	Radiographic PFS	NCT02987543
Olaparib + pembrolizumab*	Phase 1	mCRPC	After docetaxel	210	PSA response rate Safety	NCT02861573
Niraparib + radium-223	Phase 1	mCRPC	—	6	MTD	NCT03076203
Niraparib + apalutamide or abiraterone	Phase 1	mCRPC	After docetaxel	60	MTD Safety	NCT02924766

AR, androgen receptor; HRD, homologous recombination deficiency; mCRPC, metastatic castration-resistant prostate cancer; MTD, maximum tolerated dose; PARP, poly(ADP-ribose) polymerase; PFS, progression-free survival; PSA, prostate-specific antigen.

* This is a multiple-arm study testing pembrolizumab in combination with several prostate cancer therapies, including olaparib.

and progesterone receptor [PR]), the estimated pCR rates were 51% (95% Bayesian probability interval [PI], 36%-66%) in the veliparib/carboplatin arm and 26% (95% PI, 9%-43%) in the control group. It is notable that this study was not restricted to patients with DDR deficiency, although the percentage of patients in the veliparib/carboplatin arm with deleterious mutations in *BRCA1* or *BRCA2* (12/72, 17%) was higher than the percentage in the control arm (2/44, 5%). Given that platinum-based chemotherapy has shown promise in mCRPC, it would be reasonable to test platinum/PARP inhibitor combination strategies in men with advanced prostate cancer.

Although mounting evidence suggests synergistic efficacy when PARP inhibitors are combined with DNA-damaging agents, this likely comes at the expense of increased toxicity. For instance, in the aforementioned I-SPY 2 trial, grade 3 or higher neutropenia occurred in 71% of patients receiving paclitaxel in combination with veliparib and carboplatin compared with 2% in patients receiving only paclitaxel.[8] Although some of the increased bone marrow toxicity observed in the experimental arm of I-SPY 2 was likely due to the addition of carboplatin, the stark difference in the rates of neutropenia cannot be completely explained solely by the addition of carboplatin, and it seems probable that veliparib compounded this risk. Similarly, increased toxicity was observed in a randomized phase 2 study, reported by Oza and colleagues, comparing olaparib, paclitaxel, and carboplatin followed by maintenance olaparib vs paclitaxel and carboplatin alone in women with recurrent platinum-sensitive ovarian cancer.[69] This study reported grade 3 or higher neutropenia in 43% of patients receiving PARP inhibitor combination therapy and in 35% of patients receiving chemotherapy only. Larger studies are needed to better define the clinical benefit, as well as overlapping toxicity, of PARP inhibitor/chemotherapy combinations.

Homologous Recombination Deficiency and Inhibition of Androgen Receptor Signaling

Hussain and colleagues recently reported on the activity of abiraterone, a cytochrome P_{450} (CYP) 17 inhibitor able to decrease the production of androgens in extragonadal (eg, intratumoral and adrenal) sources with or without veliparib.[70] Their rationale for combining an inhibitor of androgen receptor (AR) signaling with a PARP inhibitor was based on preclinical data demonstrating that PARP is involved in the AR transcriptional machinery, and that inhibiting PARP can downregulate AR activity.[71] Randomization to this study was stratified by expression of the ETS protein as determined by immunohistochemistry (IHC) on the basis of the hypothesis that the presence of AR-regulated *ETS* oncogene fusions would predict a response to PARP inhibition. The primary endpoint was the PSA_{50} response rate (ie, the proportion of patients with decreases in PSA of ≥50% from baseline). This trial accrued 148 subjects, with 72 randomly assigned to abiraterone alone and 76 to the combination arm. The study ultimately failed to meet its primary endpoint, with similar PSA_{50} response rates in the 2 arms (63.9% with abiraterone vs 72.4% with the combination; P=.27), and ETS IHC status did not predict response to therapy. A secondary analysis involved next-generation sequencing of tumor samples (N=80) to evaluate for other genomic biomarkers that might predict response. This analysis revealed that 20 patients (25%) had alterations in HR genes (ie, *BRCA1, BRCA2, ATM, FANCA, PALB2, RAD51B,* and *RAD51C*), and interestingly, a post hoc analysis revealed that alterations in these genes predicted improved response rates irrespective of the treatment arm (PSA_{50} response rates, 58% vs 39%; P=.013).

A contemporary phase 2 study reported by Chi and colleagues tested abiraterone vs the next-generation AR antagonist enzalutamide in patients with newly diagnosed mCRPC, with crossover following PSA progression.[72] The coprimary endpoints were response and time to PSA progression following crossover. The study accrued 202 patients and randomized them equally between the groups. The PSA_{50} response rates at 12 weeks were 53% for abiraterone and 73% for enzalutamide (P=.004). Circulating cell-free tumor DNA (ctDNA) was sequenced as part of this study, and in contrast to the results reported by Hussain and colleagues, the presence of deleterious *BRCA2* or *ATM* mutations (n=14) did not predict improved outcomes. Chi and colleagues instead found an association between HRD and shorter time to progression (hazard ratio, 5.34; P<.001).

We now have 2 studies with conflicting results regarding the use of HRD to predict response to AR-signaling inhibitors. To a certain extent, the study of Chi and colleagues confirms our biases derived from natural history studies that have revealed more aggressive biology in patients with DDR alterations.[43] Caution should be exercised, however, in relying too heavily on these results. Both analyses used exploratory secondary endpoints, with relatively small subsets of patients who had HRD in each trial. The assays used in these studies were also different; Hussain and colleagues relied on tissue sequencing, whereas Chi and colleagues used newer methods to sequence selected target genes from ctDNA samples. Finally, the definitions of a DNA repair lesion in the 2 studies may have been different, in terms of both the spectrum of genes included in the biomarker panel and the designation of pathogenicity (monoallelic vs biallelic).[73] Confirmatory studies to assess the efficacy of HRD as a predictive biomarker of response/resistance to AR-signaling inhibition are therefore needed.

Targeting Mismatch Repair Deficiency and Somatic Hypermutation

The MMR pathway is responsible for correcting base-base mismatch and insertion-deletion loops, which occur during DNA replication and recombination. In tumors with MMR deficiency, long tracks of repetitive DNA sequences, known as microsatellites, are prone to strand slippage, which can result in persistent insertion-deletion loops and the rapid accumulation of mutations.[18] As such, MMR-deficient tumors have been described as exhibiting a "mutator" phenotype, which is characterized by MSI (defined as differences in microsatellite tracks between normal germline DNA and somatic tumor DNA) and somatic hypermutation (≥10 mutations per megabase of coding DNA).[74]

Lynch syndrome is a cancer predisposition syndrome characterized by germline loss of function of MMR genes and is a well-established risk factor for colorectal, endometrial, ovarian, and upper tract urothelial cancer in addition to other malignancies, including prostate cancer.[18,75] This syndrome has most commonly been associated with alterations in genes involved in the MMR pathway, including *MLH1*, *MSH2*, *MSH6,* and *PMS2*, which occur in 32%, 39%, 15%, and 14% of cases of colorectal Lynch syndrome, respectively.[76] Clinically, this syndrome can be defined with the Amsterdam criteria, in which a germline alteration in an MMR pathway gene is assumed if a family meets the following criteria: (1) 3 or more family members with a Lynch syndrome–associated cancer; (2) 2 or more successive generations affected; and (3) 1 or more family members with cancer developing before the age of 50 years.[77,78] The pathogenic role of MMR gene alterations in prostate cancer risk is not well defined, however. Pritchard and colleagues found deleterious germline MMR gene alterations in 4 of their cohort of 692 men (0.6%) with metastatic prostate cancer.[5] Estimates of MMR mutations in metastatic prostate cancer (combined somatic and germline) are likely higher, however, with series reporting mutations in anywhere from 3% to 12% of cases. Rates of MMR deficiency may be higher in more aggressive histologic subtypes.[17,79,80]

Defining the true incidence of MMR-deficient prostate cancer has been further challenged by the limitations of the assays commonly used to determine MSI status. Most MSI assays involve multiplex polymerase chain reaction (PCR) testing on a handful of genomic loci (the National Institutes of Health panel includes 5 microsatellite loci) and rely on comparisons of microsatellite loci amplified from tumors and matched normal controls.[3,75,81] The loci tested in these assays and the threshold for declaring MSI have, for the most part, been validated and optimized to detect MSI only in colorectal cancer. Because the performance of these PCR-based MSI assays for prostate cancer is unknown, clinicians should not rely too heavily on their results. Less-biased approaches for determining MSI status from next-generation sequencing data are available, and these tests may be more appropriate for noncolorectal histologies.[82]

The determination of whether an MMR gene is altered in a prostate cancer is also challenged by the fact that hypermutated prostate cancers often occur as a consequence of complex structural genomic rearrangements in MMR genes.[79,80,83] This contrasts with the inactivating mutations, loss of heterozygosity, and epigenetic silencing typical of colorectal cancers in patients with Lynch syndrome. Next-generation sequencing assays that sequence only the exons of target genes (which are the most common type of DNA-sequencing assays in clinical use) will therefore miss MMR gene alterations that arise as a result of rearrangements involving intronic regions. Assays that provide complete target gene coverage are more appropriate in this instance because they can accurately identify complex genomic rearrangements that may lead to MMR-deficient prostate cancer.[79] However, such assays are not in wide clinical use. A simpler screening approach could be to use standard IHC for MMR protein loss. For example, a recent paper used a validated IHC assay to screen 1176 primary prostate cancers for loss of MSH2, the most commonly inactivated MMR protein in prostate cancer. Although MSH2 deficiency was rare in the entire cohort (1%), MSH2 loss was enriched in patients with primary Gleason pattern 5 cancers (8%) and small cell prostate cancers (5%).[83] If these data can be replicated, screening for MSH2 inactivation in patients with primary Gleason 5 cancers and small cell prostate cancers might facilitate the identification of patients with MMR deficiency.

Because the loss of MMR gene function is often associated with a high mutational load, it has been hypothesized that individuals with this loss will have a higher tumor neoantigen burden, possibly predisposing them to respond to immune checkpoint inhibitors.[14,84-86] Proof of concept that MMR-deficient tumors may respond well to checkpoint inhibition comes from a phase 2 study that tested the anti–programmed death 1 (anti–PD-1) agent pembrolizumab in patients who had metastatic carcinomas with and without MMR deficiency (ie, MSI-high and MSI-low carcinomas, respectively).[14] In this study, 40% of the patients with MSI-high colorectal cancer had an immune-related objective response (irOR), compared with 0% of the patients with MSI-low colorectal cancer. Similarly, pembrolizumab was associated with a 50% response rate in patients with hypermutated noncolorectal gastrointestinal malignancies—supporting the hypothesis that mutational load may predict response to immune

checkpoint blockade in a range of malignancies. This study paved the way for the recent FDA approval of pembrolizumab in the treatment of patients with unresectable or metastatic MSI-high or MMR-deficient solid tumors that have progressed following prior treatment and who have no satisfactory alternative options. Of note, the approval of pembrolizumab for this indication is the FDA's first tissue-agnostic approval for a cancer therapy, which includes therapy for MMR-deficient advanced prostate cancer.[15]

Overall, immune checkpoint inhibitors have demonstrated only modest activity in unselected advanced prostate cancer, which may be a consequence of the relatively low mutational load observed in cohorts with unselected prostate cancer.[87] To date, the results of 2 phase 3 studies testing the anti–cytotoxic T-lymphocyte–associated antigen 4 (anti–CTLA-4) agent ipilimumab (Yervoy, Bristol-Meyers Squibb) in mCRPC have been negative.[88,89] Similarly, rates of response to anti–PD-1 therapy in patients with unselected prostate cancer have been low, with no responses identified in the phase 1 study of nivolumab (Opdivo, Bristol-Meyers Squibb) and an objective response to single-agent pembrolizumab in only 13% of patients.[90,91] It is worth noting, however, that a small trial testing combination enzalutamide plus pembrolizumab documented dramatic PSA declines in 3 of 10 patients.[92] In that study, 2 responders had adequate tumor material for sequencing, and one of them was found to have underlying MSI—providing a partial explanation for the high response rate observed in that study. Cases of other patients with MSI-high prostate cancer responding to PD-1 pathway inhibitors have also been reported, and studies designed to determine the rate of response to immune checkpoint inhibitors in MSI-high mCRPC are planned (Durvalumab in Treating Patients With Metastatic Hormone-Resistant Prostate Cancer; NCT02966587).[80] In another recent study, 2 of 8 patients who had mCRPC and measurable disease achieved an objective response to a combination of ipilimumab and nivolumab; neither of the 2 responding patients had MSI or hypermutation.[93]

Given that PARP inhibitors may be able to induce genomic instability, leading to neoepitope formation and enhanced sensitivity to checkpoint blockade, trials testing PARP inhibitors combined with PD-1 pathway inhibitors in advanced prostate cancer have also been launched. In an ongoing study testing the anti–programmed death ligand 1 (anti–PD-L1) agent durvalumab (Imfinzi, AstraZeneca) in combination with olaparib, 7 of 16 patients enrolled for longer than 2 months have had documented PSA_{50} responses.[94] It should be noted that although most of the patients with a PSA_{50} response had evidence of HRD, some patients with an intact HR pathway responded favorably to combination therapy. Therefore, the presence of an HRD mutation or an MMR mutation may be neither necessary nor sufficient for a response to immune checkpoint inhibitors in prostate cancer.

Conclusion

During the past few years, our understanding of the recurrent molecular alterations defining advanced prostate cancer has increased dramatically. Somewhat unexpectedly, we have learned that a significant subset of men with this disease harbor alterations in DDR pathway genes, and precision oncology strategies designed to exploit these cellular vulnerabilities are being pursued actively, including in multiple large-scale efforts aimed at developing PARP inhibitors for patients who have prostate cancer with HRD. Several retrospective reports have also shown that platinum-based chemotherapy can be highly effective in patients with HRD, which is encouraging given that these drugs are readily available.[12,62] In a similar vein, pembrolizumab has recently been approved for MSI-high or MMR-deficient advanced solid tumors, including prostate cancers, in patients who lack a reasonable alternative therapy. With this rapidly evolving treatment landscape, it is becoming increasingly important to define the genomic features of each patient's tumor so that all potentially beneficial therapies can be explored. However, as we strive toward a precision oncology framework for treating prostate cancer, critical issues surrounding the acquisition of tumor material for next-generation sequencing and the development of assays able that can accurately identify relevant somatic alterations are becoming apparent.

Currently, metastatic biopsy is the gold standard for obtaining tumor DNA for sequencing. Germline DNA assessments are insufficient because they do not capture all the relevant DDR pathway alterations used to guide therapeutic decision making. In addition, selective pressure during treatment can lead to clonal evolution, so that freshly obtained tumor DNA is preferred because it provides a snapshot of the current spectrum of mutations. Obtaining fresh tumor material is not a trivial matter, however. Prostate cancer is an osteotropic disease, and extracting DNA from osseous metastases for next-generation sequencing can be challenging.[95,96] Metastatic biopsies are also painful, potentially morbid, and expensive. Fortunately, sequencing ctDNA is quickly becoming a viable alternative.[97] These so-called liquid tumor biopsies have the advantage of allowing genomic material to be sampled easily and repeatedly as needed.

Several commercial ctDNA sequencing assays are currently available; however, caution should be exercised before blood-based assays not optimized for use on prostate cancer samples are undertaken. For example, most com-

mercially available assays are not designed to identify accurately genomic copy number changes, which are some of the most frequent alterations found in mCRPC tumors.[17] A number of groups are actively developing strategies to detect copy changes in ctDNA, and these approaches may provide a more accurate means for detecting the spectrum of mutational events that can lead to DDR pathway inactivation.[98-100] Until these technologies are widely disseminated, however, metastatic biopsy should still be considered the standard for evaluating DDR pathway alterations.

Recurrent genomic rearrangements are another hallmark of prostate cancer, and many commercial sequencing assays—based on both ctDNA and tumor tissue—do not provide sufficient gene coverage to identify such changes accurately.[101] This problem has specific relevance to MMR pathway genes because complex genomic rearrangements involving these genes have been described as a frequent cause of hypermutation in prostate cancers.[79] In addition, most PCR-based MSI assays rely on the testing of a limited number of microsatellite loci, which have been selected on the basis of data from colorectal cancer cohorts. Less-biased MSI assays that cover a larger number of microsatellite loci are currently available, however, and may be more appropriate for testing prostate cancers.[102] With the recent approval of pembrolizumab for treating MSI-high and MMR-deficient tumors, it is increasingly important to choose tests that can accurately identify these alterations across a spectrum of tumor types.

Mutations affecting DDR pathway genes are both a liability—increasing the likelihood of cancer development—and potentially a therapeutic opportunity. Bridges first described the concept of synthetic lethality in the 1920s after observing that 2 mutations were necessary to induce lethality in a fruit fly, whereas either mutation in isolation had no effect on the insect's health.[103] Only recently have we applied these principles to treating prostate cancer, developing precision oncology strategies to select patients whose tumors have lost critical DDR pathway functionality. These tailored approaches for treating patients with advanced prostate cancer have tremendous potential and should provide hope that a wave of highly effective therapies are around the corner.

Disclosures

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Personalized Medicine and Imaging

Clinical Cancer Research

MSH2 Loss in Primary Prostate Cancer

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Abstract

Purpose: Inactivation of mismatch repair (MMR) genes may predict sensitivity to immunotherapy in metastatic prostate cancers. We studied primary prostate tumors with MMR defects.

Experimental Design: A total of 1,133 primary prostatic adenocarcinomas and 43 prostatic small cell carcinomas (NEPC) were screened by MSH2 immunohistochemistry with confirmation by next-generation sequencing (NGS). Microsatellite instability (MSI) was assessed by PCR and NGS (mSINGS).

Results: Of primary adenocarcinomas and NEPC, 1.2% (14/1,176) had MSH2 loss. Overall, 8% (7/91) of adenocarcinomas with primary Gleason pattern 5 (Gleason score 9–10) had MSH2 loss compared with 0.4% (5/1,042) of tumors with any other scores ($P < 0.05$). Five percent (2/43) of NEPC had MSH2 loss. MSH2 was generally homogenously lost, suggesting it was an early/clonal event. NGS confirmed *MSH2* loss-of-function alterations in all (12/12) samples, with biallelic inactivation in 83% (10/12) and hypermutation in 83% (10/12). Overall, 61% (8/13) and 58% (7/12) of patients had definite MSI by PCR and mSINGS, respectively. Three patients (25%) had germline mutations in *MSH2*. Tumors with MSH2 loss had a higher density of infiltrating $CD8^+$ lymphocytes compared with grade-matched controls without MSH2 loss (390 vs. 76 cells/mm^2; $P = 0.008$), and $CD8^+$ density was correlated with mutation burden among cases with MSH2 loss ($r = 0.72$, $P = 0.005$). T-cell receptor sequencing on a subset revealed a trend toward higher clonality in cases versus controls.

Conclusions: Loss of MSH2 protein is correlated with *MSH2* inactivation, hypermutation, and higher tumor-infiltrating lymphocyte density, and appears most common among very high-grade primary tumors, for which routine screening may be warranted if validated in additional cohorts. *Clin Cancer Res; 23(22); 6863–74. ©2017 AACR.*

Introduction

Approximately 10% of advanced/metastatic prostate tumors have a markedly elevated rate of single-nucleotide mutations (1, 2), almost always due to underlying somatic and/or germline inactivation of genes in the mismatch repair (MMR) family (*MSH2*, *MSH6*, *MLH1*, or *PMS2*) and often accompanied by microsatellite instability (MSI; ref. 1), similar to what has been observed in colorectal carcinoma (3). Similarly, a significant fraction of the commonly used prostate cancer cell lines have biallelic loss of MMR genes, including DU145 (4, 5), LNCaP (5–7), CWR22RV1 (8), and VCaP cells (8). Taken together, this work in advanced tumors and cell lines suggests that the rate of MMR defects in prostate cancers may be similar to the prevalence seen in colorectal carcinoma (~15% of cases). Importantly, advanced prostate tumors with MMR gene loss and hypermutation may respond favorably to immunotherapies targeted to PD-1 (9, 10) and/or CTLA-4, similar to what has been seen in colorectal carcinoma, due to the generation of neoepitopes and resulting immune recognition of "non-self" tumor antigens (11, 12).

Although previous studies have focused on MMR defects in advanced prostate cancer, the relative frequency and clinical significance of MMR alterations in primary prostate cancer is less certain. Most studies describing the prevalence of microsatellite instability in primary prostate cancer were performed more than a decade ago and a wide range of MSI frequency (2%–65%) has been reported (13–15). The numbers and types of microsatellite markers used to define MSI in these older studies differed significantly from international standardized guidelines subsequently developed for MSI testing in colorectal carcinomas (16, 17). When current MSI definitions are super-imposed on these earlier studies, the MSI prevalence in prostate cancers is rarely higher than 10% overall (18). Indeed, more recent work using the previously recommended mono- and di-nucleotide marker panels from the Bethesda Consensus Panel (16, 17) has suggested that the rate of MSI in primary prostate tumors is <4% (19) similar to recent genomic profiling studies of primary prostate cancer where the rate of MMR gene loss was even lower, <3% (20). Even rarer, recent studies of Lynch syndrome, an autosomal-dominant

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Translational Relevance

Inactivation of mismatch repair (MMR) genes is associated with microsatellite instability (MSI) and hypermutation in metastatic prostate cancers and may predict response to immunotherapy. To screen for MMR defects in primary prostate cancers, in which alterations are rare and standard DNA sequencing may miss complex rearrangements, we used an immunohistochemistry assay for MSH2. We find that MSH2 loss is enriched among primary tumors with high-grade histology, is an early and clonal event, and is highly predictive of underlying *MSH2* genomic alteration, hypermutation, and high $CD8^+$ lymphocyte density. In contrast to observations in colorectal carcinoma, only about half of primary prostate tumors with *MSH2* inactivation have evidence of MSI by PCR and/or next-generation sequencing assays using traditional cutoffs. These data have implications for the testing of primary tumor specimens for MMR defects in the setting of metastatic prostate cancer for which pembrolizumab may be a treatment option following recent FDA approval.

condition associated with increased incidence of early colorectal and endometrial carcinomas due to germline MMR gene inactivation, have suggested that increased risk of prostate carcinoma is likely part of the syndrome (21–28), though not all studies are consistent (29, 30). Small series of Lynch syndrome–associated prostate cancer patients have found that some, though notably not all, prostate tumors arising in this setting are associated with MSI and there may be an association with increased tumor-infiltrating lymphocytes and higher pathologic grade (21, 26).

Given the relative rarity of MSI and MMR gene alterations in primary prostate cancers, few studies have characterized primary prostate tumors with MMR gene inactivation outside of Lynch syndrome. This is of particular interest and clinical relevance with the recent FDA-approval of the PD-1 inhibitor pembrolizumab to treat metastatic tumors of all histologic types with MMR deficiency or MSI. To identify and molecularly characterize primary prostate tumors with sporadic and/or germline MMR defects, we utilized an IHC assay for MSH2. We initially focused on MSH2 because this MMR protein was the most robustly expressed in primary prostate tumors, is the most commonly altered MMR gene in advanced prostate cancer (1, 20), and the MMR gene most frequently implicated in Lynch syndrome patients who develop microsatellite-unstable prostate cancer (21–26). Screening for MSH2 loss by immunohistochemistry (IHC) is particularly useful in the setting of primary prostate cancer, as it can be easily applied to large numbers of tumors and large tumor areas to screen for the relatively rare tumors with protein loss. In addition, it is potentially more sensitive than standard whole-exome or targeted sequencing protocols, which may miss the complex genomic rearrangements that commonly involve MMR genes in prostate cancer (1). Herein, we pathologically and molecularly characterize primary prostate tumors with MSH2 protein loss.

Materials and Methods

Patients and tissue samples

In accordance with the US Common Rule and after institutional review board (IRB) approval, a total of 8 partially overlapping tissue microarray (TMA) cohorts containing a total of 1290 (n = 1,133 unique) samples of prostatic adenocarcinomas from radical prostatectomies performed at Johns Hopkins were queried using MSH2 IHC. Most of these cohorts have been previously described, and notably many were created to enrich for adverse oncologic outcomes, so they do not represent an unbiased survey of a radical prostatectomy population. In brief, these consisted of: (i) a cohort of consecutive tumors at radical prostatectomy from 2000 to 2004, including all tumors with Gleason score >6 (n = 462 samples; ref. 31); (ii) a cohort of high-grade (Gleason score 9/10) tumors at radical prostatectomy from 1998 to 2005, designed for comparison with high-grade urothelial carcinomas (n = 28; ref. 32); (iii) a cohort of all radical prostatectomies from 2004 to 2014 with primary Gleason pattern 5 and available clinical follow-up (n = 71); (iv) a cohort of African-American radical prostatectomy samples from 2005 to 2010, all with Gleason score 4 + 3 = 7 and higher (n = 84; ref. 31); (v) a cohort of patients who all developed metastatic disease and were treated with abiraterone/enzalutamide after radical prostatectomy at Johns Hopkins from 1995 to 2011 (n = 34); (vi) a cohort of patients with ductal adenocarcinoma and/or cribriform Gleason score 8 adenocarcinoma at radical prostatectomy from 1984 to 2004 (n = 46; ref. 33); (vii) a case–cohort study of men undergoing radical prostatectomy from 1992 to 2009 who subsequently developed metastatic disease (n = 325; ref. 34); and (viii) a cohort of men with biochemical recurrence following radical prostatectomy from 1992 to 2009 (n = 240; ref. 35); (9) finally, a separate cohort of 43 neuroendocrine prostate carcinomas (NEPC) with confirmed small cell carcinoma histology on TMA was also queried by MSH2 IHC (36). Additional control tissues were procured from a radical prostatectomy sample from a patient with a known pathogenic germline mutation in *MSH2*, as well as from an additional 10 prostatectomy specimens with tumors with primary Gleason pattern 5 but intact MSH2 immunostaining.

Finally, electropherograms from an additional 10 cases of colorectal carcinoma that were MSI-H by PCR and tested within the last year were utilized to compare differences in microsatellite marker shifts between prostate and colorectal carcinoma with MMR defects.

Cell line TMA

Fifty-six cell lines from the NCI-60 cell line panel (Developmental Therapeutics Program, NCI) were used to evaluate MSH2 IHC staining. All cell lines were pelleted, fixed in 10% neutral buffered formalin, and processed and cut as tissue. Cell lines were punched and tissue microarrays created as described previously (37). Short tandem repeat genotyping was completed once prior to creation of the cell line TMA.

Mismatch repair protein IHC and interpretation

MMR protein IHC was performed on the Ventana Benchmark autostaining system utilizing primary antibodies from Ventana (Roche/Ventana Medical Systems). MSH2 IHC used a mouse mAb (clone G219-1129), MSH6 IHC used a mouse mAb (clone 44), MLH1 IHC used a mouse mAb (clone M1), and PMS2 IHC used a rabbit mAb (clone EPR3947). All samples were incubated with primary antibody after antigen retrieval in CC1 buffer, and primary antibody incubation was followed by detection with the UltraView HRP system (Roche/Ventana Medical Systems). Each tissue microarray spot or standard histologic section containing tumor cells was visually dichotomously scored for presence or

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absence of cytoplasmic MMR protein signal by a urologic pathologist blinded to the sequencing/MSI testing data (TLL). A spot was considered to show MMR protein loss if any tumor cells in any tumor spot showed MMR protein loss, with intact staining in admixed benign prostate glands and/or surrounding stromal cells, endothelial cells, or lymphocytes. Spots without internal control staining were considered ambiguous and not scored. All samples were initially screened for MSH2 loss by scoring TMA spots; however, for all cases with MSH2 loss on TMA, confirmatory immunostaining for MSH2, MSH6, MLH1, and PMS2 was also performed on standard histologic tissue sections.

DNA isolation

For samples from the TMAs, a total of five 0.6-μm punches were procured from the same tumor and benign areas in the paraffin block sampled on the TMA. For standard histologic sections, tumor and normal tissue was macrodissected guided by hematoxylin and eosin–stained section. DNA was extracted from FFPE material using the Qiagen FFPE DNA extraction kit according to the manufacturer's instructions. DNA concentrations were quantified with the Qubit fluorometer, using a Quant-iT dsDNA High Sensitivity Assay Kit (Invitrogen).

PCR-based microsatellite instability analysis

Microsatellite instability (MSI) analyses were carried out using multiplex PCR with fluorescently labeled primers, included in the MSI Analysis System, Version 1.2 (Promega Corp.), for amplification of five mononucleotide repeat markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27) and two pentanucleotide repeat loci (Penta-C and Penta-D) to confirm identity between the tumor and benign tissue pair. The PCR reactions were performed in samples containing at least 250 ng of DNA, 0.05 U/μL TaqGold (Applied Biosystems), and sterile dH_2O (Sigma). The PCR was performed using a Veriti Thermal Cycler (Thermo Fisher Scientific) using the following program: 95°C 11 minutes, 96°C for 1 minute, 10 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 70°C for 1 minute; 20 cycles of 90°C for 30 seconds, 58°C for 30 seconds, 70°C for 1 minute; and 60°C for 30 minutes. PCR products were mixed with formamide and size standard, denatured, and run on an ABI 3130 capillary electrophoresis instrument using injection times of 30–180 seconds. Cancers were designated MSI-H with 2 shifts, MSI-L with 1 shift, and MSS with no shifts relative to the germline pattern. The pattern and number of bases shifted were compared with the first 10 MSI-H colorectal cancers diagnosed in 2016. Bimodal and trimodal patterns consisted of one or two additional (nongermline peaks), where the novel peak was distinct in that bases in between it and the germline peak had lower fluorescent intensity. Shoulder pattern had an extension of peaks (bases of equal or lower intensity) beyond those that could be attributed to germline peaks injected for different times. In some cases, we observed the presence of single base shifts in peaks of one of the markers without any further changes in other markers, and these cases were not classified as unstable.

Targeted next-generation sequencing and MSI by NGS

Targeted next-generation deep sequencing of MMR genes and MSI by NGS (mSINGS) analysis was performed using UW-OncoPlex (http://web.labmed.washington.edu/tests/genetics/UW-OncoPlex) as described previously (38, 39). UW-OncoPlex is a clinically validated assay performed in the CLIA-laboratory setting that sequences to 500× average depth all exons, introns, and flanking regions of *MSH2*, *MSH6*, and *MLH1* and all exons of *PMS2* and *EPCAM*. Genomic libraries were made from 1 μg of genomic DNA extracted from prostate tumor and matched normal (germline) formalin-fixed paraffin embedded tissue and a custom Agilent SureSelect XT capture set used for target enrichment. After target enrichment and barcoding, libraries were pooled and sequenced on an Illumina NextSeq 500 instrument with paired-end 101-bp reads. A custom bioinformatics pipeline detects single nucleotide variants, indels of all sizes, structural rearrangements, *PMS2* pseudogene disambiguation, and copy number changes. mSINGS analysis was performed on UW-OncoPlex data as previously described using a total of 65 mononucleotide microsatellite loci (40). Total mutation burden was estimated from targeted sequencing data as previously described with a threshold of 12 coding mutations/Mb for hypermutation (39, 41). Sequencing interpretation was done by an expert molecular pathologist (C.C. Pritchard) who was blinded to clinical data and other molecular testing results.

CD3, CD8, and PD-L1 immunostaining and digital image quantification

CD8 and CD3 immunostaining was performed on standard histologic slides in a CLIA-accredited laboratory using a mouse mAb for CD8 (clone C8/C8144B, 760-4250; Cell Marque) and rabbit polyclonal antibody for CD3 (A0452, Dako/Agilent Technologies) with antigen detection by the Ventana iView system (Roche/Ventana Medical Systems). PD-L1 immunostaining was performed using a rabbit mAb (SP142, Ventana) on the Ventana Benchmark platform, also using standard histologic sections. For image analysis of CD8 immunostaining, a single standard histologic slide stained with CD8 was scanned at 20× magnification on the Aperio Scanscope AT Turbo (Leica). $CD8^+$ and $CD3^+$ cells per millimeter squared tissue were quantitatively performed with the Aperio Digital Pathology software (Leica). For each immunostained standard slide, all tumor tissue present, excluding benign epithelium, with minimal intervening stromal tissue was selected for analysis. An average of 67 mm^2 of tumor tissue area was selected for analysis (range: 16–152 mm^2). $CD8^+$ or $CD3^+$ cells within the selected tumor area were identified by Aperio software as described previously (42), and the ratio of $CD8^+$ or $CD3^+$ cells to the total tumor area analyzed was calculated for each case. PD-L1 staining was scored as positive if >1% of immune cells or tumor cells showed PD-L1 membranous positivity.

T-cell receptor sequencing

T-cell receptor sequencing (TCR-seq) of the CDR3-variable region of the T-cell receptor β chain was performed as described previously (43) on a subset of 6 samples (3 cases with MSH2 loss and 3 primary Gleason pattern 5 controls; Adaptive Biotechnologies). Briefly 2–3 μg of DNA was prepared as described above from tumor samples using macrodissection of standard histologic sections. Once prepared, DNA was transferred to Adaptive Technologies for sequencing. TCR metrics and clonality indices were calculated using the ImmunoSeq Analyzer (44).

Statistical analysis

Statistical analysis was performed using Student *t* test, Fisher exact test, and linear regression. *P* values of <0.05 were considered statistically significant.

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Results

Initial validation of MSH2 IHC using prostate cancer cell lines and tumor tissues with known *MSH2*-mutant genomic status

For initial validation, we performed MSH2 IHC on prostate cancer cell lines with and without known alterations in MSH2 (Supplementary Fig. S1). DU145 cells have a heterozygous splice site mutation in *MLH1* with missense mutation in the other genes (4, 5) and had intact staining for MSH2 and MSH6, with loss of MLH1 and PMS2 as expected. PC3 cells have intact MMR genes by sequencing (8) and by IHC. LNCaP cells have a homozygous deletion of *MSH2* and *MSH6* (5–7), and showed loss of MSH2 and MSH6 immunostaining. VCaP cells have a heterozygous frameshift mutation in *MSH6* (c.1085; ref. 8) and showed intact MSH2 immunostaining. Finally, CWR22RV1 cells have a homozygous deletion of *MSH2* and *MSH6* (8) and showed loss of MSH2 and MSH6 staining, with intact staining for MLH1 and PMS2. To begin to assess the assay in primary prostate tumor samples, we utilized a radical prostatectomy sample from a patient with a known germline pathogenic mutation in *MSH2* (p.A636P; refs. 45, 46) and somatic loss of heterozygosity (i.e., confirmed biallelic inactivation), which was MSI-high (MSI-H) by PCR (3/5 markers shifted) and MSI-positive by mSINGS (though notably without evidence of clear-cut hypermutation, at only 9 mutations/Mb, possibly due to low tumor DNA content). In this sample, MSH2 and MSH6 protein expression was entirely absent by IHC (Supplementary Fig. S1).

Clinicopathologic features of cases with MSH2 loss by IHC

Next, we screened for MSH2 protein loss in tissue microarray spots from a total of 1,176 unique primary prostate carcinomas, including 1,133 prostatic adenocarcinomas and 43 prostatic small cell neuroendocrine carcinomas (NEPC). Altogether, 1.2% (14/1,176) of prostate primaries had MSH2 loss, including 1% (12/1,133) of primary adenocarcinomas and 5% (2/43) of NEPC cases (Figs. 1 and 2). Clinicopathologic characteristics of these cases are detailed in Table 1 and the Gleason grade distribution of the adenocarcinomas queried by MSH2 immunostaining is detailed in Supplementary Table S1. The average patient age of cases with MSH2 loss was 62 years, which was not significantly different from the overall cohort of 1,176 cases (59 years; $P = 0.53$). Tumors with MSH2 loss were generally extremely aggressive by pathologic features, including tumor grade (Supplementary Fig. S2) and stage. Overall, 71% (10/14) of the cases with MSH2 loss were either Gleason score 9 adenocarcinomas or NEPC cases. The four remaining cases included one case of Gleason score 8, and three with Gleason score 7 (see Table 1 for breakdown), although one had tertiary Gleason pattern 5 cancer. Of the 12 adenocarcinoma cases at radical prostatectomy that had pathologic stage information available, 50% (6/12) were pathologic stage pT3b or higher (two with nodal involvement), 33% (4/12) were pT3a and 17% (2/12) were pT2. When adenocarcinomas were analyzed separately, 8% (7/91) of tumors with primary Gleason pattern 5 ($5 + 4 = 9$ or $5 + 5 = 10$) cases had MSH2 loss compared with less than 1% of tumors with all other grades ($P < 0.0001$). Interestingly, there seemed to be a much greater enrichment for MSH2 loss among primary Gleason pattern 5 cases, even when compared with Gleason score $4 + 5 = 9$ cases (7/91 vs. 1/108; $P = 0.02$).

For cases with MSH2 loss by TMA screening, confirmation of loss was performed on standard histologic sections, along with

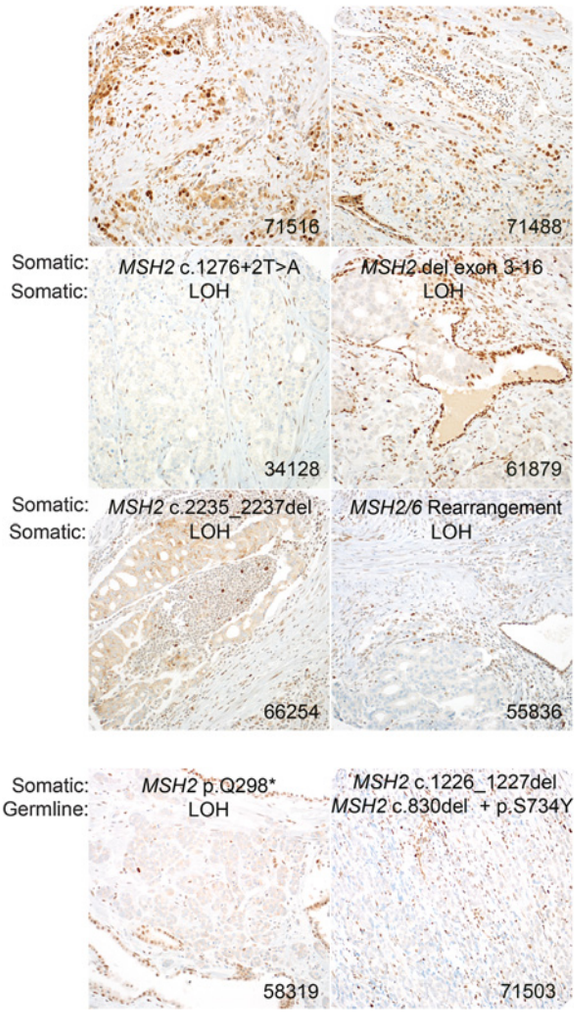

Figure 1.

Representative MSH2 immunostaining in formalin-fixed paraffin-embedded primary prostate tumors with biallelic *MSH2* inactivation. Top row: Gleason score $5 + 4 = 9$ prostate tumors with intact nuclear immunostaining and wild-type *MSH2* gene. Second and third rows: tumors with loss of MSH2 expression and somatic two-copy *MSH2* genomic inactivation. Although in some sections a weakly positive cytoplasmic stain of unknown significance can be observed, the nuclei remain negative in all tumor cells, with intact staining in stromal cells, lymphocytes, and benign epithelium in all cases as an internal positive control. Bottom row: representative MSH2 immunostaining in formalin-fixed paraffin-embedded primary prostate tumors with germline and somatic *MSH2* gene inactivation. Both tumors lack nuclear staining for MSH2. Adjacent benign prostatic glands and stromal cells maintain nuclear expression of MSH2 as an internal control. All photomicrographs are reduced from 200×.

immunostaining for MSH6, MLH1, and PMS2. All cases with MSH2 loss showed concordant MSH6 loss, as expected because stability of the proteins is only ensured as heterodimers, with intact MLH1 and PMS2 (Supplementary Figs. S1 and S3). Notably, staining for MSH6 in stromal cells was often quite weak and focal, making it difficult to use this stain to screen large numbers of cases for MSH6 loss in tumor cells (Supplementary Fig. S3). When all cases were evaluated on standard histologic slides, MSH2 staining was homogenously lost in all tumor cells sampled in

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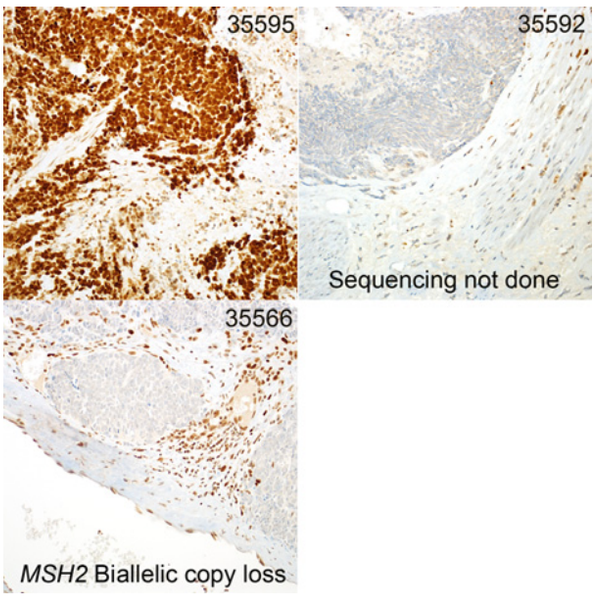

Figure 2.

Representative MSH2 immunostaining in formalin-fixed paraffin-embedded small cell neuroendocrine carcinoma (NEPC) of the prostate. Standard histologic tissue sections of a small cell carcinoma (35595) shows robust MSH2 nuclear staining, whereas two other small cell carcinoma tumors (35592 and 35566) lack nuclear staining with intact stromal and lymphocyte staining. All photomicrographs are reduced from 200×.

the dominant tumor nodule from each case, suggesting that it was an early and clonal event in the evolution of the tumor. This is in stark contrast to other genomic alterations that we have profiled *in situ*, such as *PTEN* deletion (47).

MSH2 sequencing

To confirm that our immunoassay was detecting underlying genomic alterations at the *MSH2* locus, we analyzed normal and tumor DNA from all cases with MSH2 loss using a targeted next-generation sequencing (NGS) assay specifically designed to detect somatic/germline mutations as well as small and large-scale genomic rearrangements at the MMR gene loci (38). We did not sequence unselected (i.e., MSH2-intact) cases in this study as nearly 500 cases of primary prostate cancer have been sequenced to date in the TCGA effort, with excellent representation of Gleason score 9 tumors (20). In these studies, the median mutation burden has been less than 1 mutation/Mb of coding DNA, regardless of tumor grade, with only 1% of unselected primary tumors showing genomic alterations in *MSH2*. In our cases with MSH2 loss, NGS confirmed (at least monoallelic) *MSH2* loss-of-function alterations in all (12/12) samples with adequate tumor DNA available for analysis. Two cases did not have enough DNA for sequencing. Definite evidence of biallelic inactivation was present in 83% (10/12) of cases, with the 2 cases that lacked evidence of biallelic deletion both showing low tumor content, which can make loss-of-heterozygosity calls challenging from sequencing data; one of these two cases showed possible LOH. Cases without apparent biallelic inactivation were indistinguishable from cases with two-copy loss of *MSH2* based on MSH2 and MSH6 immunostaining (Supplementary Fig. S4), suggesting that loss of the second copy was likely present but not detected by sequencing. Somatic and germline alterations are described in Table 2. Overall, 25% (3/12) of cases showed somatic large-scale deletions and/or genomic rearrangements involving both the *MSH2* and *MSH6* loci, including one case with a deletion involving *MSH2* exons 3–16 and all of MSH6, one case with *MSH2* biallelic copy loss and another case with a large-scale rearrangement involving both loci, including a 5.7 Mb inversion (Fig. 1). All of these cases demonstrated loss of heterozygosity. The remaining cases showed predominantly small deletions resulting in frameshift or splice site alterations in *MSH2*, with a rare missense mutation known to affect splicing (p.G669V) (Fig. 1). Overall, 25% (3/12) of cases showed germline pathogenic lesions in *MSH2*, including a frameshift, a splice site and a nonsense mutation (Fig. 1). Two of these cases had somatic loss of heterozygosity or other somatic inactivation consistent with a second hit to the gene in the tumor DNA only, and another case had likely loss of heterozygosity. Only one of the three patients with germline *MSH2* inactivation had a documented history of Lynch syndrome with a prior colorectal carcinoma and upper tract urothelial carcinoma. Two other patients had no known history of Lynch syndrome, although one had a prior colorectal carcinoma and both had a strong family history of colorectal and other Lynch syndrome–associated carcinomas.

Table 1. Clinicopathologic characteristics of primary prostate tumors with MSH2 loss by IHC

Block ID	TMA	Specimen type	Tissue type	Year	Age	Race	Gland weight	Gleason primary	Gleason secondary	Gleason sum	Path stage	Known Lynch syndrome?
58319	1	RP	AdCa	2001	48	W	38	3	4	7 (tertiary 5)	T3AN0	Yes
66254	3	RP	AdCa	2009	63	W	52.4	5	4	9	T3BN0	No
19236	6	RP	AdCa	2001	64	W	52	4	4	8	T3AN0	No
55795	1	RP	AdCa	2003	63	W	56	4	5	9	T2N0	No
34128	1	RP	AdCa	2001	65	W	40	4	3	7	T3BN0	No
55836	3	RP	AdCa	2005	65	W	69.6	5	4	9	T2N0	No
71503	3	RP	AdCa	2011	69	W	43.7	5	4	9	T3AN0	No
61879	3	RP	AdCa	2014	58	W	56.4	5	4	9	T3BN0	No
35566	9	TURP	NEPC	2001	72			NA	NA	NA		No
22966	2	RP	AdCa	2005	63	W	84.8	5	4	9	T3BN1	No
71484	3	RP	AdCa	2005	66	W	35	5	4	9	T3AN0	No
35592/3	9	TURP	NEPC	2007	79			NA	NA	NA		No
60913	3	RP	AdCa	2010	47	H	66	5	4	9	T3BN1	No
3131	7	RP	AdCa	1993	56	W	54.8	3	4	7	T3BN0	No
22533	Control	RP	AdCa	1993	55	W	60.4	5	4	9	T3BN0	No

Abbreviations: AdCa, adenocarcinoma; NEPC, neuroendocrine prostate cancer; RP, radical prostatectomy; TURP, transurethral resection of the prostate.

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Table 2. Molecular characteristics of primary prostate tumors with MSH2 loss by IHC

ID	Somatic MMR alteration(s)	LOH	Germline MMR status	Known Lynch syndrome?	MSI-PCR	MS markers shifted	MSI (mSINGS)	Hyper-mutation	Total mutation burden[a]	Mutations/ Mb Coding	Other mutations found	CD8/mm^2	PD-L1 +
58319	LOH	Yes	*MSH2* c.892C>T (p.Q298*)	Yes	MSS	0 of 4	IND (15%)	No	13	10	*CHEK2* (p.W93Gfs*17); *EPHB6* (p.L881Cfs*39); *NF2* (p.R336Q); *FANCA* (exon 3-6del?)	145	No
66254	*MSH2* c.2235_2237del (p.I747del)	Yes	None	No	MSI-H	2 of 4	IND (15%)	Yes	45	34	*PBRM1* (p.R58*); *ARID1A* (p.R1223C, p.K1072Nfs*21); *TP53* (p.R306*)	535	Yes
19236	*MSH2* c.1728del (p.I577Lfs*13)	No[b]	None	No	MSS	0 of 5	NEG[b]	No[b]	3	3	*SPOP* (p.F102V)	83	No
55795	*MSH2* c.1613del (p.N538Tfs*5) + c.547C>T (p.Q183*)	No[b]	None	No	MSI-H	2 of 4	NEG[b]	Yes[b]	36	28	*AR* (p.R727H)	85	No
34128	*MSH2* c.1276+2T>A (splicing)	Yes	None	No	MSI-H	2 of 4	POS	Yes	16	13	*MSH6* (p.F1088Lfs*5); *ARAF* (p.R103W); *GNAS* (p.R81M); *CDK8* (p.R356*)	350	Yes
55836	*MSH2/6* locus rearrangement (5.7Mb inversion)	Yes	None	No	Fail	NA	POS	Yes	31	24	*MSH6* (exon 3-6del?); *FOXA1* (p.H247Y, p.M59I); *ARID1A* (p.R1074W, p.R1733Q)	633	No
71503	*MSH2* c.830del (p.L277*) + c.2201C>A (p.S734Y)	No	*MSH2* c.1226_1227del (p.Q409Rfs*7)	No	MSS	0 of 5	POS	Yes (ultra)	138	104	*MSH6* (p.N534Efs*4); *POLD1* (p.D402N)	1,016	Yes
61879	*MSH2* exon 3-16 *MSH6* del	Yes	None	No	MSI-H	3 of 4	POS	Yes	101	76	*PIK3CA* (p.E726K, p.H1047R, p.E81K)	523	Yes
35566	*MSH2* biallelic copy loss	Yes	NA	No	MSS	0 of 4	POS	Yes	26	20	*RB1* (p.R73Sfs*36); *TP53* (p.R175H); *RB1* (p.R73Sfs*36)	22	NA
22966	None	Possible	*MSH2* c.942+3 A>T (splice site mutation)	No	MSI-H	4 of 4	POS	Yes	59	45	*PTEN* (p.R173C, p.R130Q); *TP53* (p.R342*)	1,020	Yes
71484	*MSH2* c.2006G>T (p.G669V) + c.943-10T>A (splicing)	No[b]	None	No	MSS[c]	0 of 4	IND (18%)[b]	Yes[b]	31	24	None	331	No
35592/3	ND	ND	ND	No	MSI-H	2 of 4	ND	ND	ND	ND	ND	114	Yes
60913	*MSH2* c.646-2A>G (splicing)	Yes	None	No	MSI-H	2 of 4	POS	Yes	35	27	*CSF1R* (p.W839*, p.W839*); *PIK3CA* (p.H1047R); *PTEN* (p.R233*)	527	Yes
3131	ND	ND	ND	No	MSI-L[c]	1 of 3	ND	ND	ND	ND	ND	70	No
22533 (control)	LOH	Yes	*MSH2* c.1906G>C (p.A636P)	No	MSI-H	3 of 5	POS	No[b]	11	9	*TMPRSS2* (p.A347Lfs*5)	72	ND

Abbreviation: NA, not assessed.
[a]Coding only out of 1.3 Mb.
[b]Tumor content <20%.
[c]Low amplification.

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PCR-based microsatellite instability

Thirteen of the fourteen cases with MSH2 protein loss had interpretable MSI testing by PCR; one case failed MSI testing in several replicates, likely due to the presence of PCR inhibitors. Overall, only 61% (8/13) of these had evidence of MSI by PCR, although analysis was frequently limited by low overall DNA amplification level (48, 49). Of those classified as unstable by the PCR assay, 7 of 8 had 2 or more microsatellite markers with signs of instability (MSI-H) and 1/8 had only one shifted marker (MSI-L), although this case had low amplification.

Among the prostate cases with evidence of MSI, there were discrete bimodal peak shifts of 2–6 bases (mean 4 bases) and a high prevalence of shoulder pattern shifts (13/21 or 62% of unstable loci had a shoulder pattern with remaining unstable loci showing a bimodal pattern; Fig. 3; Supplementary Table S2). These findings were notably more subtle than those seen in 10 colorectal cancer controls with MSI-H, where peak shifts of 4–13 bases were observed (mean 7 bases), with a predominance of bimodal and trimodal shifts in all peaks (only 3/47 or 6% of unstable loci showed a shoulder pattern, with 72% showing a bimodal pattern and 21% showing a trimodal pattern). Among prostate cases, there was no apparent predominance of shifts in one marker over the other. There was notable failure of amplification of the BAT-26 marker in most (11/14) samples, possibly due to the presence of amplification inhibitors in the FFPE-extracted DNA. The presence of 4 amplified markers is still sufficient to make MSI calls if there is presence of 2 or more markers demonstrating MSI (16). In one case, however, there was one shifted marker (BAT-25) among 3 amplified ones, this case was considered MSI-L.

mSINGS

Twelve of the fourteen cases with MSH2 protein loss had adequate DNA available for sequencing. Overall, only 58% (7/12) cases had definite evidence of MSI by mSINGS at a cutoff of >20% unstable loci (38), although analysis was frequently limited by low tumor content in the analyzed DNA, which must be above 20% for this validated assay (Table 2; Supplementary Table S3). Among the 5 cases that did not have definitive MSI by mSINGS, three were indeterminate (one of which had inadequate tumor purity), and two were negative (both of which had inadequate tumor content). Cases that were MSI-H by PCR were likely to be MSI by mSINGS. Of the cases that were MSI-H by PCR assay with sequencing data, 67% (4/6) were positive for MSI by mSINGS, with one case that was negative by mSINGS but with inadequate tumor purity, and one case slightly below threshold for calling MSI by mSINGS (15% of loci queries, scored as indeterminate). Interestingly, cases that were MSS by PCR were also likely to be positive or indeterminate for MSI by mSINGS. Of the cases that were MSS by PCR assay, 40% (2/5) were positive for MSI by mSINGS and 40% (2/5) were indeterminate by mSINGS with evidence of MSI at 18% and 15% of loci queried. The remaining microsatellite stable (MSS) case by PCR was negative for MSI by mSINGS, but showed low tumor content.

Mutation burden

Hypermutation, defined as more than 12 mutations per Mb on the 1.3 Mb NGS panel, was present in 83% (10/12) of tumors with MSH2 loss by immunostaining, and cases had a median of 26 (range: 3–104) mutations/Mb. The case with the highest

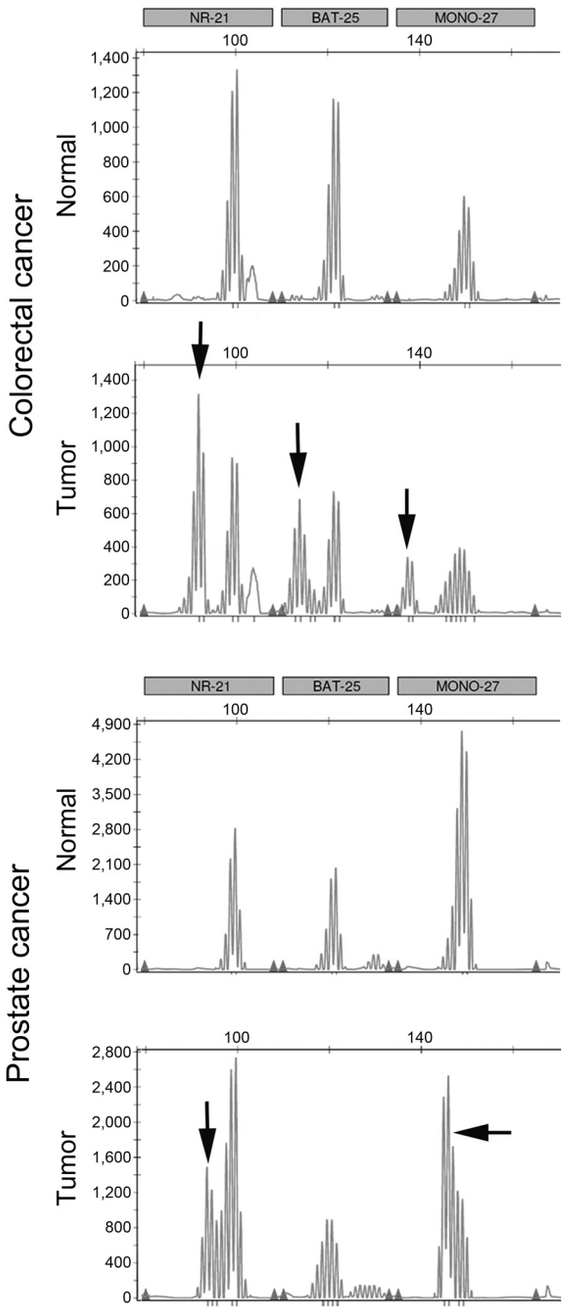

Figure 3.

Representative electropherograms of colorectal carcinoma and prostatic adenocarcinoma cases that are MSI-H. MSI-PCR testing (Promega panel) for representative colorectal carcinoma and primary prostate carcinoma samples. Colorectal tumor sample shows a clear bimodal pattern with distinct peak shifts in NR-21, BAT-25, and MONO-27 mononucleotide markers (new peaks present in tumor sample but absent in normal sample are indicated by vertical arrows). In contrast, the MSI prostate tumor sample shows a bimodal shift in NR-21 of only six bases (indicated by vertical arrow) and a subtle shift of MONO-27 ("shoulder" morphology, indicated by horizontal arrow).

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mutation burden (104 mutations/Mb, considered to be ultra-mutated) had an additional somatic mutation in *POLD1* involving the exonuclease "proofreading" domain (p.D402N) that likely contributed to the ultra-high mutation burden. The patient with the lowest mutation burden (3 mutations/Mb) was also negative for MSI by mSINGS and was MSS by MSI-PCR, though the mSINGS result was limited by low tumor content.

Infiltrating lymphocyte quantification and TCR-seq

Tumor-infiltrating lymphocytes (TIL) appeared increased by hematoxylin and eosin staining in many cases with MSH2 protein loss, though there was notable variability (Supplementary Fig. S2). By immunostaining, we digitally quantified the number of $CD3^+$ (Fig. 4A) and $CD8^+$ TILs (Fig. 4B) in each case with MSH2 loss and 10 primary Gleason pattern 5 cases without MSH2

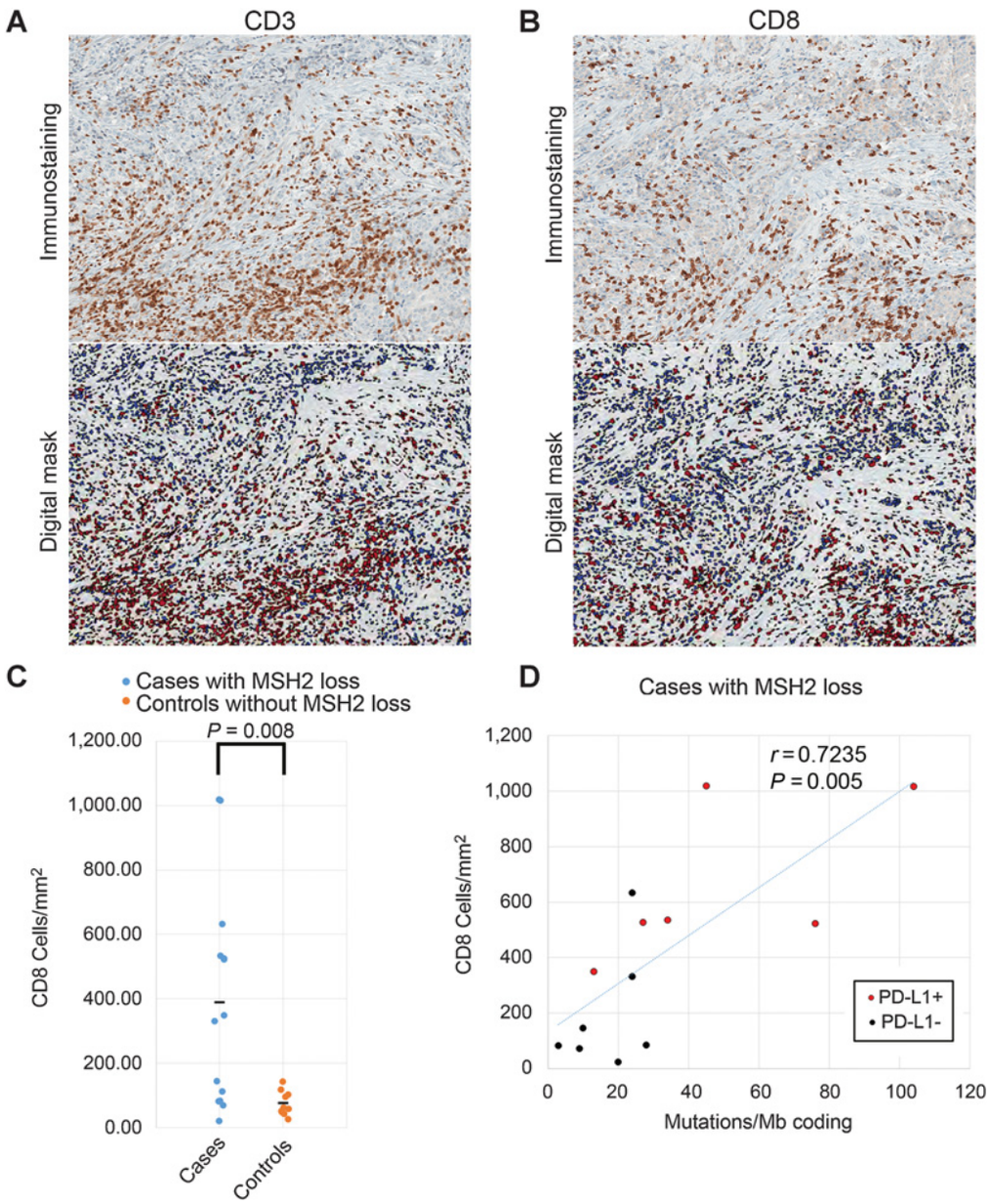

Figure 4.

$CD8^+$ tumor-infiltrating lymphocyte density in primary prostate tumors with MSH2 loss. **A,** Immunostaining for CD3 identifies a high number of tumor-infiltrating lymphocytes in a prostate tumor with MSH2 loss, case 71503 (top). Aperio image analysis software is useful to identify $CD3^+$ cells (red) in selected tumor regions and surrounding tumor and stromal nuclei (blue; bottom). **B,** Immunostaining for CD8 identifies a high number of tumor-infiltrating lymphocytes in a prostate tumor with MSH2 loss, case 71503 (top). Aperio image analysis software is useful to identify $CD8^+$ cells (red) in selected tumor regions and surrounding nuclei (blue; bottom). CD8 and CD3 cell counts were highly correlated in all cases with MSH2 loss and controls without MSH2 loss. **C,** Mean density of $CD8^+$ infiltrating lymphocytes are significantly higher in cases with MSH2 loss compared with matched control tumors with MSH2 intact and primary Gleason pattern 5. **D,** Density of $CD8^+$ infiltrating lymphocytes is significantly correlated with mutation burden among tumors with MSH2 loss.

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protein loss using a single standard histologic section of tumor for each radical prostatectomy. $CD3^+$ and $CD8^+$ lymphocyte density (quantified on adjacent tissue sections) were highly correlated across cases ($r = 0.94$) and controls ($r = 0.84$), thus we focused on the $CD8^+$ fraction in further analysis (Fig. 4B). There was a mean of 390 $CD8^+$ cells/mm^2 among the cases with MSH2 loss, significantly higher than the mean of 76 $CD8^+$ cells/mm^2 seen among the 10 grade-matched control cases ($P = 0.008$, Fig. 4C). Similarly, the CD8 to CD3 cell ratio was significantly higher among cases (mean = 0.59) compared with controls (mean = 0.29, $P < 0.001$), which together with the increased absolute number of $CD8^+$ cells, suggests a more prominent cytotoxic lymphocytic response among the tumors with MSH2 loss compared with those without. Clinicopathologic variables or the presence of an underlying germline alteration in *MSH2* did not correlate appreciably with the number of $CD3^+$ or $CD8^+$ cells/mm^2, as some cases with germline alterations had very high lymphocyte counts and some had quite low counts. Similarly, the presence of biallelic MSH2 inactivation and MSI status of the tumor by either PCR or sequencing did not show obvious association with lymphocyte count. Strikingly, however, the quantitative $CD8^+$ lymphocyte density was significantly correlated with the overall mutation burden among the 12 cases with MSH2 loss and available sequencing data ($r = 0.7235$, $P = 0.005$, Spearman correlation coefficient, Fig. 4D). PD-L1 staining (defined as the presence of >1% positive cells among immune cells or tumor cells) was positive in 50% (7/14) of tumors with MSH2 loss; however, positivity was most commonly seen in the immune cell compartment (Supplementary Fig. S5). PD-L1–positive cases tended to have higher lymphocyte counts (and mutation burden) overall (Fig. 4D), with one notable exception seen in an NEPC case with low lymphocyte counts where sequencing data was not available. TCR-seq was performed on a small subset of 3 cases and 3 controls with adequate DNA and relatively lower tissue block age per recommendations that blocks less than 5–10 years of age be utilized for this assay (Supplementary Table S4). As expected given the differences in lymphocyte counts, the mean number of templates available for sequencing was higher in the cases compared with the controls (10,590 vs. 4,628). There was a trend towards a higher mean productive clonality (0.079 vs. 0.042) in cases compared with controls, although this did not reach statistical significance in this small sample size. Notably, there was marked variation among the cases with MSH2 loss in terms of productive clonality indices (0.043 to 0.117) that was not obviously correlated with any other genomic or lymphocyte metrics.

Discussion

The findings in the current study support the concept that MSH2 protein loss, as measured by IHC, is highly correlated with underlying genomic inactivation of *MSH2* and hypermutation. Our study is among the first to compare contemporary MSH2 IHC to next-generation sequencing in primary prostate tumors (9), and the first to do so in a large number of specimens. Use of this IHC assay enabled us to screen >1,100 primary tumors to identify the relatively rare cases with MMR defects, comprising only about 1% of cases our cohorts. Accordingly, this is among the first studies to examine the phenotype of sporadic primary prostate tumors with MMR defects. Perhaps the most interesting phenotypic correlation discovered here is that MSH2 loss appears more common among very-high-grade prostatic primary tumors, with rates approaching 10% among tumors with primary Gleason pattern 5 in our series. These data are particularly striking as we only queried one of four genes known to be involved in MMR, suggesting that the true rate of MMR gene alterations in this population is very likely to be even higher. Clearly, given the small cohort examined, additional validation studies are required to confirm this association. However, these findings are generally consistent with previous reports of high-grade prostate cancer in Lynch syndrome patients, particularly among those with MSI (21, 26, 50). If validated in subsequent studies, these data argue for routine clinical screening of very-high-risk patients for germline and sporadic MMR gene loss using IHC or other techniques.

The high Gleason grade of most tumors with MSH2 loss, combined with the overall enrichment of MMR defects among metastatic compared to primary cases, suggests that these tumors may behave aggressively from the outset, in contrast to what has been observed in MMR-defective colorectal cancers. Many of the prostate tumors with MSH2 loss in our study had significantly increased $CD8^+$ lymphocyte density. The presence of a marked lymphocytic infiltrate, which is also frequently seen in colorectal tumors with MMR loss, may contribute to the undifferentiated, high-grade appearance of the tumor in some cases (51). This phenomenon is also commonly seen in lymphoepithelioma-like carcinomas (52) and medullary tumors of the breast (53), which are not associated with MMR defects and in all of these cases, the presence of high-grade carcinoma may not always be well-correlated with aggressive tumor progression. However, beyond the appearance of high histologic grade, the potentially aggressive behavior of primary prostate tumors with MSH2 loss was also supported by their generally high pathologic stage in the current series. It may also be consistent with the relatively higher rate of MMR defects among advanced or metastatic prostate cancer cases (1, 2) compared with primary tumors (20), as well as the enrichment of MMR defects observed in aggressive variants of prostate cancer, including ductal adenocarcinoma (9) and potentially NEPC. Unfortunately, we had insufficient clinical follow-up data and biased selection of tumors for screening in the current study, both of which precluded comparison of long-term oncologic outcomes among cases with MSH2 loss and those with intact MMR. This will be the focus of future studies.

Our use of an *in situ* assay to examine MSH2 status led to the observation that MSH2 protein loss is almost always homogeneous within a given tumor nodule. This is notable, given the fact that only a minority of our cases had germline alterations in *MSH2*, and suggests that biallelic somatic inactivation of MSH2 is frequently an early clonal event when it occurs. This is in stark contrast to other common genomic alterations in primary prostate cancer, such as *PTEN* deletion or *TP53* mutation, which are also enriched in metastatic and castration-resistant disease (47, 54, 55) and manifest a much more heterogeneous staining pattern in the primary tumor. Although we did select for cases with more homogeneous alterations in MSH2 by screening for loss using tissue microarray (TMA) punches, PTEN heterogeneity may be easily captured in TMA punches (47, 54), suggesting that this was not likely a major confounder.

In our cohort with MSH2 protein and genomic loss, the MSI PCR assay was substantially less sensitive for MSH2 loss than has been previously described for other tumor types. MSI PCR testing is generally approximately 95% sensitive for underlying genomic alteration in *MSH2* in colorectal carcinoma meta-analyses (56). Rare discordant cases generally show intact IHC, with evidence of

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MSI by PCR, often due to functionally deleterious missense mutations that fail to compromise protein expression. However, cases of colorectal carcinoma with clear genomic loss by DNA sequencing but absence of MSI by PCR is extraordinarily rare to our knowledge. Similarly, high concordance of MSI PCR and MSH2 IHC has also been observed in endometrial carcinomas (57). In contrast, among our prostatic primaries, only 61% (8/13) of cases with MSH2 protein loss had evidence of MSI by PCR, including one case which was unstable at only one microsatellite, consistent with MSI-L status. The low sensitivity of traditional MSI markers in primary prostate carcinoma is paralleled by the more subtle peak shifts observed in prostate tumors in our study, compared with those typically seen in colorectal carcinoma. Although studies in colorectal carcinoma are abundant (56), few contemporary studies have compared MMR IHC assays or genomic testing to MSI PCR results in primary prostate cancer outside of the context of Lynch syndrome, and older studies have shown only weak correlations (58). In a more recent study of Lynch syndrome patients, only 66% (4/6) of prostatic adenocarcinomas with MSH2/6 protein loss showed evidence of MSI by PCR-based testing; however, this study did not use the contemporary Promega 5 marker microsatellite panel (17, 48, 50). In a second study, 88% (7/8) of Lynch prostatic carcinomas with MSH2/6 protein loss showed evidence of MSI by PCR-based testing, although it is notable that 5 of 7 of the cases with MSI were categorized as MSI-L, meaning only one of five markers was unstable (26). Similar to our results in the current study, these data suggest that contemporary PCR panels may be inadequate to screen for MMR defects in primary prostate cancer.

There is emerging evidence that MSI testing by next-generation sequencing is at least as, and potentially more, sensitive for MSI than traditional PCR-based testing (40). MSI testing by sequencing interrogates a much larger panel of microsatellite loci than PCR testing, which could increase sensitivity. In addition, the 5 mononucleotide repeat markers that make up the standard MSI PCR testing panel were largely designed for detection of MSI in colorectal carcinoma, and perhaps are not optimized for similar studies in prostate carcinoma where alternative microsatellites may be more sensitive markers of MSI. However, using previously established cutoffs of 20% of unstable loci to call MSI, mSINGS did not have a markedly different sensitivity for cases with MSH2 protein loss than PCR testing (58% vs. 61%) in our study; however, these data are limited by the low tumor content (below the 20% cutoff) in 25% of our samples (including the only samples that were entirely negative for MSI by mSINGS). In addition, we had a number of indeterminate cases, with MSI at some loci but not reaching the 20% threshold, such that decreasing the threshold to 15% of tested loci with instability was sufficient to raise the sensitivity to 83%. Further optimization of NGS MSI assays are needed, and should ideally be performed on samples with a high tumor content.

Regardless, both the mSINGS data and MSI-PCR results seem to point to a similar conclusion that MSI in primary prostate cancer is likely more subtle and difficult to detect compared with that seen in colorectal cancer. The reasons for this difference remain unclear. It is possible that prostate samples have relatively lower tumor content compared with colorectal tumor samples, which can decrease the sensitivity of MSI testing by both methods. It is also tempting to speculate that the relatively low proliferation and apoptosis rates in primary prostate cancer may be one contributing factor. As MSI increases over time with errors accrued after each cell division, and the absolute proliferation rate in primary prostate cancer is generally lower than that in colorectal cancer, tumors from the prostate (even if of equal size to those in the colon) may have undergone markedly fewer cell divisions, contributing to the lower level of MSI in these prostate tumors. Consistent with this hypothesis, MSI PCR assays were much more concordant with underlying MMR gene genomic status in advanced metastatic prostate cancer than we found in our primary tumors (1), perhaps suggesting that more extended genomic evolution is required for manifestation of the MSI phenotype.

In this context, hypermutation may be a more sensitive marker of underlying *MSH2* genomic loss than MSI testing in our cohort, as hypermutation was present in all but two of the cases with MSH2 loss (83%). There were some cases with hypermutation in the absence of MSI, suggesting that hypermutation may precede, or perhaps occur in the absence of, MSI in primary prostate cancer, and that this might be the more sensitive marker of underlying MMR defects in primary prostate cancer. Remarkably, the mutation burden in tumors with MSH2 loss was highly correlated with infiltrating lymphocyte density, a finding that potentially corroborates the anecdotal response of these tumors to immunotherapy (9, 10). Overall, both the absolute number of $CD8^+$ lymphocytes was increased among tumors with MSH2 loss, as well as the relative proportion of the $CD3^+$ cells that were cytotoxic T cells (the CD8/CD3 ratio). Although the prognostic significance of this ratio is unclear, these data are consistent with a more prominent cytotoxic T-cell response among the MSH2-null tumors in our cohort. However, more detailed additional immunophenotypic studies are required to definitively test this. Importantly, however, there was a wide variation in both mutation burden and the lymphocytic response among prostate primaries with MSH2 loss, and this variability was not easily explained by underlying genomic alteration in *MSH2*. Future studies will examine whether mutation burden and/or lymphocyte density or clonality index by TCR-seq are predictive biomarkers for duration of response to immune checkpoint blockade in the prostate and other organs.

Our study has some important limitations. First, we focused on only a single MMR protein, MSH2, for validation. This was in large part because protein expression of MSH6, MLH1, and PMS2 appeared to be considerably weaker than MSH2 expression in the prostate using IHC assays validated for colorectal carcinoma (see MSH6 in Supplementary Fig. S3); we are currently working to further optimize these assays for screening similar to what we did with MSH2. In addition, loss of MSH2 is most common in prostate cancer compared with MSH6, MLH1, and PMS2 (1, 2, 20). However, this single assay will clearly lack sensitivity for screening prostate tumors for MMR defects as it will miss alterations in the other MMR genes. Because of the design of our study, we also cannot give an accurate estimate of the true prevalence of MSH2 loss in unselected primary prostate cancers. Although we screened >1,100 primary tumors for loss, many of these cases were selected for inclusion on TMAs designed to enrich for adverse oncologic outcomes, which may confound our prevalence estimates. Future studies in high-risk populations where sequencing is performed on all tumors screened by IHC will be useful to address prevalence and IHC assay sensitivity questions.

Collectively, our data have important implications for screening algorithms used to identify prostate cancer patients that may benefit from immune checkpoint blockade. Although it remains debated, our cases add additional evidence that prostate cancer is, definitively, a Lynch syndrome–associated tumor. Our study

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suggests that MMR gene alterations are commonly clonal and homogenous in primary prostate tumors, which should facilitate screening of primary tumor samples (even those collected on needle biopsies) for MSH2 deficiency, and suggests that heterogeneity between metastases is likely to be rare (although differences in MSI and hypermutation status are possible). In addition, we demonstrate that, pending validation in independent cohorts, the highest rates of MSH2 loss are among tumors with the most aggressive pathologic features, namely primary Gleason pattern 5 and neuroendocrine prostate carcinomas. Given the generally poor oncologic outcomes in these groups, these data suggest that screening this population routinely for MMR defects may be useful, perhaps even at diagnosis, to potentially direct patients toward immunotherapy. The relatively subtle MSI by PCR assays in many primary prostate tumors with genomic *MSH2* loss is intriguing and indicates that MSI PCR using the contemporary markers developed for colorectal carcinoma may be an inadequate test in isolation for primary prostate carcinomas. Indeed, screening by next-generation sequencing for hypermutation may be among the most sensitive genomic tests in this context and since tumor infiltrating $CD8^+$ cell density is highly correlated with mutation burden, this may also provide an additional screening tool for labs that do not have ready access to sequencing. Finally, assessing for MMR protein loss by IHC remains an excellent and relatively inexpensive test to screen for underlying genomic alterations in MMR genes, especially if future studies can optimize and validate MSH6, MLH1, and PMS2 IHC assays. Ultimately, these IHC assays may be paired with mutation burden analysis for routine screening of high-risk populations and to stratify patients for clinical trials of immune checkpoint blockade therapy.

Disclosure of Potential Conflicts of Interest

J.R. Eshleman reports receiving speakers bureau honoraria from Merck. T.L. Lotan reports receiving commercial research grants from Ventana. No potential conflicts of interest were disclosed by the other authors.

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Clinical Cancer Research

MSH2 Loss in Primary Prostate Cancer

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Case Report

Durable Response to Immune Checkpoint Blockade in a Platinum-Refractory Patient With Nonseminomatous Germ Cell Tumor

Ethan A. Chi, Michael T. Schweizer

Clinical Practice Points

- Some germ cell tumors might respond favorably to immune checkpoint blockade.
- Clinical trials to test immunotherapeutic approaches in chemorefractory germ cell tumors are warranted.

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Keywords: Anti-PD1, Anti-PDL1, Immunotherapy, Nivolumab, Testicular cancer

Introduction

Metastatic germ cell tumors are often curable with platinum-based chemotherapy.[1] Even in those with disease relapse after first-line therapy, some can still be salvaged with second- or third-line therapies, including high-dose chemotherapy followed by tandem autologous stem cell transplantation (auto-SCT).[2-4] However, for patients with disease progression after auto-SCT, long-term disease control is rare, and these patients are often left with palliative chemotherapy as their only option.[5] The lack of effective therapies available to these chemorefractory patients represents a major unmet medical need, and therapies that exert their antitumor effect through mechanisms distinct from standard cytotoxic agents are desperately needed. As a class, immunotherapies hold great promise in this regard.

The major role of the adaptive immune system is to recognize and destroy cells with foreign antigens, including tumor cells. A clinically relevant mechanism by which cancers are able to escape T-cell mediated destruction is through stimulating the programmed death 1 (PD1) pathway—a negative feedback system that represses cytotoxic immune responses.[6] Recent advances have shown that blocking this pathway with antibodies directed toward PD1 or its ligand (PDL1) can produce remarkable clinical responses in an array of malignancies.[7-12] In this report we describe the case of a man with a poor-risk nonseminomatous germ cell tumor (NSGCT) who had disease progression after auto-SCT, and now shows a durable response to the immune checkpoint inhibitor nivolumab.

Case

The patient initially presented with back pain, and was subsequently found to have lung, bone, and retroperitoneal metastases. Laboratory results revealed a β-human chorionic gonadotropin (HCG) > 210,000 mIU/mL, and metastatic biopsy showed a NSGCT (choriocarcinoma). He subsequently received 4 cycles of VIP (etoposide, ifosfamide, and cisplatin) followed by orchiectomy.[13] Shortly after completing chemotherapy he was found to have brain metastases, which were treated with stereotactic radiosurgery (SRS). Within 6 months he developed a central nervous system disease recurrence, which was also treated with SRS. He was then referred for high-dose chemotherapy and tandem auto-SCT. After auto-SCT, surveillance scans showed a new lung metastasis, which was resected before initiating palliative gemcitabine and oxaliplatin treatment.[14-16] Seven months later he developed another brain metastasis, which was resected. Postcraniotomy markers failed to normalize and he developed progressive pulmonary metastases.

At this point, the patient transferred care to our center. Targeted next-generation sequencing (UW-OncoPlex, University of Washington, Seattle, WA) on the craniotomy specimen revealed copy number gains in portions of chromosome 1 and copy losses on portions of chromosomes 1, 8, and 17.[17] He was initially referred for consideration of a phase I study; however, because of his history of recurrent brain metastases, his clinical trial options were limited. We therefore opted to initiate off-label nivolumab treatment, and shortly after initiating immunotherapy his tumor markers began to decline. A new brain metastasis was identified 5 months after starting nivolumab treatment, and this was treated with SRS. Surveillance scans have since shown a partial radiographic response and

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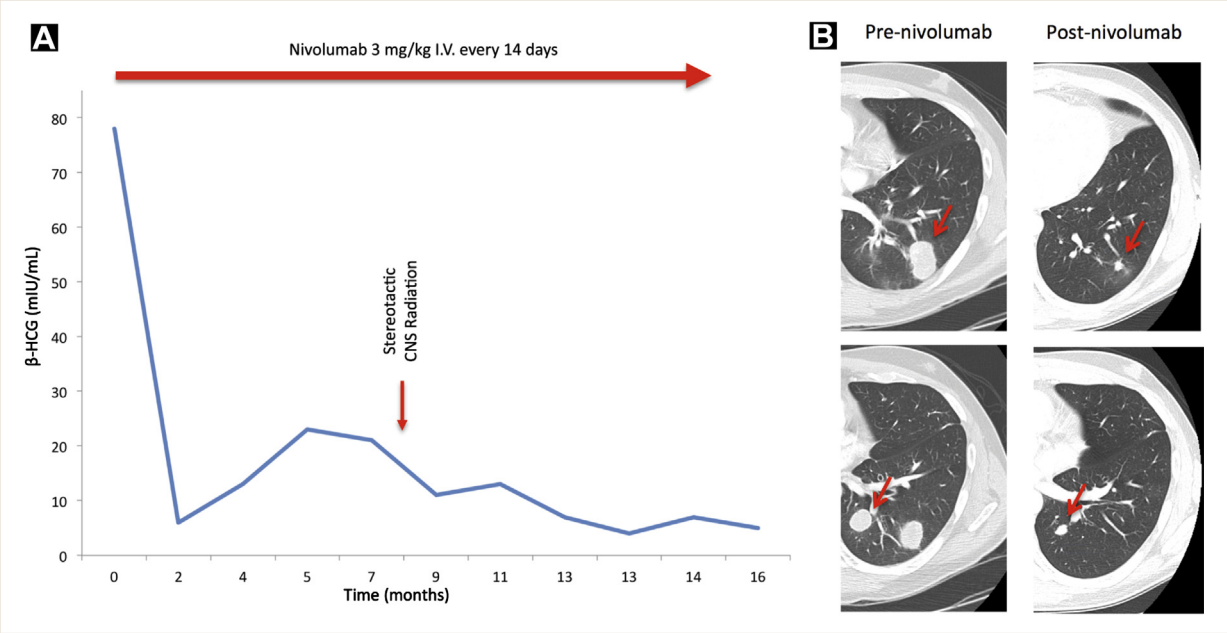
Figure 1 (A) β-human chorionic gonadotropin (HCG) Trend During Nivolumab Treatment 3 mg/kg I.V. Every 2 Weeks. (B) Representative Computed Tomography Images From the Patient at Baseline (Pre-Nivolumab) and After 14 Months of Nivolumab (Post-Nivolumab) Treatment. Red Arrows Indicate Index Lesions



β-HCG has stabilized (Figure 1). After month 14 of treatment, the patient transitioned to watchful waiting, with continued disease stability at short-term follow-up.

Discussion

To date, there has been minimal data published on the use of immune checkpoint inhibitors in patients with metastatic germ cell tumors. One case report documented a partial radiographic response after a single dose of nivolumab. This individual was initially diagnosed with melanoma on the basis of fine-needle aspirate; however, after core biopsy was reclassified as having an embryonal carcinoma. This change in diagnosis prompted a switch to standard platinum-based chemotherapy.[18] In another case series, 7 patients with platinum-refractory NSGCT were treated with anti-PD1 therapy.[19] Four patients had rapid disease progression and died shortly after starting treatment. Three others continued to receive therapy for at least 6 months, with only 1 patient (embryonal NSGCT) achieving a partial radiographic response.

Although previous reports have only documented objective radiographic responses to anti-PD1 therapy in those with embryonal histology, the patient presented in this report provides evidence that choriocarcinomas might also respond favorably to immune checkpoint blockade. Interestingly, this patient was found to have several genomic copy number changes, consistent with previous reports; however, there were no clear mutational events that could explain his sensitivity to immunotherapy.[20] As it stands, multiple germ cell tumor histologies have been shown to respond to PD1 pathway suppression, and prospective trials to evaluate immune checkpoint inhibitors in patients with refractory germ cell tumors appear warranted. These studies should prospectively evaluate if histologic subtype or other candidate biomarkers (eg, PDL1 expression, mutation load) correlate with clinical benefit.[6]

Disclosure

The authors have stated that they have no conflicts of interest.

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